CELL SURFACE STRUCTURES INVOLVED IN THE INDUCTION OF MACROPHAGE ACTIVATION FOR TUMOR CELL KILLING

By

MARK PATRICK HAYES

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MARK PATRICK HAYES

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Major Department: Pathology

Macrophage activation for tumor cell killing proceeds through a series of stages induced by independent stimuli. Gamma interferon is a lymphokine which primes macrophages to respond to a second, triggering signal for complete activation to the cytolytic stage. Purified, recombinant-derived murine gamma interferon (MuIFN-gamma) was radiolabelled with ^{125}I and used to determine binding parameters on various macrophage populations. A single class of specific, high affinity binding sites was revealed on bone marrow culture-derived and peritoneal macrophages, with a Kd from 4×10^{-10} to 7×10^{-9} M, depending upon the MuIFN-gamma used for each experiment, and $2-4 \times 10^{4}$ sites/cell. Macrophages with altered responsiveness to

activating stimuli did not express these differences at the MuIFN-gamma receptor level. However, priming of macrophages with MuIFN-gamma resulted in a significant down-regulation of receptor expression (50% decrease in receptor number). Treatment of macrophages with proteases revealed that the MuIFN-gamma receptor was resistant to trypsin and chymotrypsin, but not to Pronase. Receptors could be synthesized de novo and replaced on the cell surface in 4 hours following removal of Pronase. These cells also recovered the ability to respond to MuIFN-gamma and LPS in the same time period. Trypsin affected the ability of macrophages to respond to LPS and heat-killed Listeria monocytogenes, and permitted the distinction between LPS/HKLM and polyinosinic-polycytidylic acid triggering responses (the latter was trypsin-resistant). Trypsin treatment of macrophages revealed the dissociation of the triggering response from the induction of proteins by these stimuli. Further studies were directed toward solubilization and enrichment of the MuIFN-gamma receptor. Plasma membranes prepared from WEHI-3 cells retained specific high affinity MuIFN-gamma binding sites. Membranes were solubilized with octyl-β-D-glucopyranoside. Solubilized receptor, assayed by a liposome precipitation method, retained the properties of the membrane form, with a slight decrease in affinity. Substantial enrichment of receptors was achieved by chromatography on immobilized

MuIFN-gamma. These results have enhanced the understanding of interactions of activating stimuli with macrophages and provide the groundwork for further structural and functional studies of the MuIFN-gamma receptor.

CHAPTER I

INTRODUCTION

The Mononuclear Phagocyte System: Differentiation and Modulation

The cells comprising the mononuclear phagocyte system possess a remarkable spectrum of functional capacities, most of which are important for the variety of roles that these cells play in host defense (reviewed in 190). The mononuclear phagocyte system refers to the lineage of cells that ultimately gives rise to circulating blood monocytes and macrophages that exist in almost every tissue of the body. The earliest recognized cell in this lineage is the monoblast, which derives from a multipotential stem cell in the bone marrow. Monoblasts mature in the marrow through promonocytes to monocytes which are released into the circulation; the circulating monocytes then migrate into the tissues and complete their differentiation into macrophages (189). The positive control of mononuclear phagocyte differentiation is primarily maintained by the colony-stimulating factors (CSF's). Those affecting macrophage development are interleukin-3 (IL-3; also known as multi-CSF), granulocyte-macrophage colony-stimulating factor, and macrophage colony-stimulating factor (also

known as CSF-1) (112). The tissue macrophage is the terminal differentiation stage of mononuclear phagocytes, but monocytes and macrophages are subject to modulation of their phenotype by a variety of mediators in their environment. The functional plasticity of macrophages in response to microenvironmental stimuli is a hallmark of the mononuclear phagocyte system.

The functional versatility of macrophages has become apparent over the last three decades, with the realization that macrophages are not only phagocytic scavengers with the capacity for exceptional directed motility, but also act as secretory cells for a variety of important molecules and as effector cells involved in immune responses, antimicrobial activity, and the control of neoplasia (190). The most distinguishing characteristic of macrophages is their phagocytic activity, first described by Metchnikoff, who observed their capacity for ingestion of microbial organisms (113). Even unstimulated macrophages (if such a population actually exists) maintain an impressive rate of fluid phase pinocytosis, turning over the equivalent of their cell surface area every 33 minutes (179). Macrophages are equally efficient at the phagocytosis of particulate materials, both nonspecifically and via immunologically specific recognition structures on the cell surface (immune phagocytosis) (168). These structures include receptors for the Fc region of immunoglobulins and

for complement components (105). The regulation of phagocytosis, therefore, can occur at two levels: 1) control of nonspecific endocytic activity and 2) modulation of the receptors involved in immune phagocytosis.

In addition to endocytosis, macrophages exhibit several other complex functional capacities that are instrumental for host defense. These include chemotaxis, antigen presentation, and antimicrobial and antitumor activities. Chemotaxis is regulated by gradients of chemoattractant molecules that bind to receptors on the macrophage cell surface (171). The most well-characterized of these attractants are the N-formylated oligopeptides; the other known attractants are the complement cleavage product, C5a; the arachidonic acid metabolite, leukotriene B_4; and a less well-defined mediator termed lymphocyte-derived chemotactic factor (LDCF) (172). In addition to chemoattractants, there also exist inhibitors of macrophage migration; a lymphocyte-derived mediator termed migration inhibitory factor (MIF) that causes macrophages to accumulate in its presence was first described in 1966 (37). Antigen presentation by macrophages is of primary importance in the generation of immune responses (reviewed in 188). The processing and presentation of antigens requires the expression of Class II major histocompatibility antigens on the macrophage cell surface. This expression is not constitutive, but is regulated by the

micro-environment of the macrophage (33). The modulation of Class II antigen expression is primarily mediated by T lymphocytes (14,15,158,178,195), although some regulation of Ia expression in the mouse appears to be T cell-independent (100).

The antimicrobial and antitumor activities of macrophages are complex functions that normally do not reach their full potential unless the macrophage has been stimulated or "activated" by factors in its environment. The concept of macrophage activation has been loosely defined since its inception by Mackaness in the 1960's (101,102). The definition of an activated macrophage depended largely upon the function(s) that were being examined (30,37,123). Most of the classical studies of murine macrophages utilized cells derived from the peritoneal cavity; in unmanipulated mice, these are referred to as <u>resident</u> macrophages (30). When mice are injected intraperitoneally with a sterile irritant such as thioglycollate broth, proteose peptone, or bacterial lipopolysaccharide, macrophages derived from these cavities exhibit numerous phenotypic differences when compared to their resident counterparts; these cells have been termed <u>inflammatory</u> (108), <u>stimulated</u> (68), or <u>responsive</u> (1) macrophages. The heightened activities of these macrophages presumably results from their exposure to a largely undefined mixture of inflammatory mediators. Macrophages

are also substantially affected by immunological mediators. This was the phenomenon first reported by Mackaness (101,102), who observed that acquired resistance to microorganisms depended upon immune sensitization, but was mediated nonspecifically by activated macrophages. It quickly became evident that T lymphocyte interactions were instrumental in stimulating macrophage activity (120), particularly with respect to increased microbicidal capacity (18,88,169). The importance of soluble T lymphocyte products, termed lymphokines (42), in the modulation of macrophage antimicrobial activity became apparent (6,20,51,85,122).

Inflammatory macrophages and lymphokine-stimulated macrophages both exhibit distinct alterations in many host defense functions. Inflammatory macrophages have substantially increased endocytic activity when compared to resident macrophages (43), and also develop the capacity to ingest complement (C3b)-coated erythrocytes (111) (C3b-mediated phagocytosis is virtually nonexistent in resident cells). Fc-mediated phagocytosis was shown to be enhanced by lymphokines (193). Lymphokine supernatants were also found to contain Ia-inducing factor(s) that were active both in vivo (158) and in vitro (15,178,195). An examination of the antimicrobial and antitumor activities of macrophages eventually resulted in an understanding of the stepwise nature of macrophage activation, i.e., that

resident macrophages or monocytes proceed through a sequence of stages toward activation (30). The second step is represented by the inflammatory macrophage, which becomes more responsive to lymphokine-mediated activation than resident macrophages (94,149,181). Activation of macrophages is a highly regulated phenomenon that is critical to the appropriate response of these cells for host defense functions.

Macrophage Activation for Tumor Cell Killing

The antitumor activity of macrophages has been the subject of intense investigation since the initial observations by Hibbs et al., that macrophages derived from toxoplasma-infected mice had the capacity to kill tumor cells nonspecifically (67). Susceptibility of target cells to killing by these macrophages correlated with high saturation density of growth (no contact inhibition), lectin agglutinability, and tumorigenicity (66).

The activation of macrophages for tumor cell killing was shown to be mediated by lymphocyte products; a lymphokine activity termed macrophage activating factor (MAF) was first described by Piessens et al. (137). Lymphokines referred to as macrophage cytotoxicity factor (MCF) (99) and specific macrophage arming factor (SMAF) (47) were independently described in the supernatant fluids of appropriately stimulated lymphocytes. The latter factor

(SMAF) was unlike other described MAF's in that it possessed specificity for the tumor cells used to generate it. No further characterization of SMAF has appeared since its original description. All of the macrophage activating factors were determined to be products of T lymphocytes. Closer examination of the activation phenomenon revealed that MAF did not fully activate macrophages for tumoricidal capacity under conditions that were free of endotoxin contamination (130,199). This discovery led to the definition of two more stages in the activation sequence (68,108,150). Macrophages exposed to MAF-containing lymphokines were referred to as primed, based on their hyper-responsiveness to a second, triggering stimulus, most commonly supplied by bacterial lipopolysaccharide (LPS). These observations characterized a four-stage sequence of activation: (1) the unstimulated, resident macrophage, (2) the stimulated or inflammatory macrophage that is more responsive to lymphokines, (3) the primed, noncytolytic macrophage, characterized by its sensitivity to triggering agents, and (4) the activated, cytolytic macrophage. These stages have been characterized in vivo and in vitro (68,108,150). The effects of lymphokine and LPS are synergistic, i.e., responses to their combinations are far greater than expected additive effects (68,108,130). In most strains of mice, this synergy is extreme: macrophages will not become tumoricidal when stimulated with

lymphokines alone unless trace amounts of LPS are present. Increasing the amount of LPS decreases the requirement for lymphokine, and the converse is also true (130). The sequence of stimulation is also important. Macrophages stimulated with lymphokines prior to addition of LPS become cytotoxic, but if LPS is added prior to lymphokines, no tumoricidal activity develops (134). This reinforces the notion that priming results in a macrophage alteration that enhances its responsiveness to the triggering agent (in this case, LPS) prior to the addition of that agent. Other substances that have been found to act as triggering agents for macrophage activation include double-stranded RNA (185), maleylated and acetylated proteins (74), heat-killed Listeria monocytogenes organisms (165), and muramyl dipeptide (154), as well as lymphokine components (108).

The stages of macrophage activation outlined above have all been characterized by various functional and molecular markers (30,73,103). Cohn established multiple alterations that exist in the inflammatory or responsive stage of activation: increased spreading, increases in endocytic activity, ATP utilization, superoxide anion production, secretory enzymes (most notably plasminogen activator), and lysosomal hydrolases, and decreased 5'-nucleotidase activity (30). Johnson et al. distinguished the primed stage by the capacity to specifically bind tumor cells and the fully activated stage by the secretion of a

putative cytolytic protease (73). More definitive biochemical markers were characterized by MacKay and Russell (103). Analysis of newly synthesized proteins by macrophages at various stages of activation by two-dimensional gel electrophoresis distinguished specific protein phenotypes induced by priming and triggering stimuli. Specifically, a 47,000-dalton protein that appeared only in macrophages that were primed (designated p47b), and a pair of proteins of 71,000 and 73,000 daltons that appeared only in macrophages that were treated with triggering agents (designated p71,73), were consistently observed under these respective conditions; concomitant expression of both p47b and p71,73 was always associated with the activated state (i.e., tumoricidal activity) (103).

Much progress has been made in the molecular characterization of macrophage-activating factor(s) since the initial descriptions by Nathan et al. (120) and Fowles et al. (51). They described responses such as enhanced spreading, motility, phagocytosis, and glucose oxidation (120) as well as increased bacteriostasis (51). Piessens et al. (137) were the first to describe a factor that activated macrophages for tumor cell killing. Confusion as to the precise nature of MAF has largely been due to the imprecise definition of macrophage activation. Restriction of this definition to the function of priming macrophages for tumor cell killing allowed the eventual discovery that

at least one important MAF produced by stimulated T lymphocytes is gamma interferon (reviewed in 160). Early studies of the physicochemical nature of MAF produced in stimulated spleen cell cultures indicated a molecular weight of 55,000 daltons by gel filtration, sensitivity to protease and pH below 4.0 or above 10.0, and adsorption to immobilized Cibacron Blue (95). Four years later, spleen cell-derived MAF was found to be inseparable from IFN-gamma by pH and temperature sensitivity, immunoreactivity (antiserum to IFN-gamma), and chromatography on Sephacryl S-200 (82), Con A-Sepharose, polynucleotide columns, and HPLC gel permeation columns (148). Biosynthesis and secretion of MAF was coincidental with that of IFN-gamma by T-cell lines and clones derived from them (79,140). The use of T-cell hybridomas was instrumental in confirming that IFN-gamma is a MAF (162). Using one such hybridoma, the laboratories of Schreiber (164) and Russell (132) demonstrated the identity of MAF and IFN-gamma. High resolution chromatographic techniques (HPLC chromatofocusing and gel filtration) were unable to distinguish the two activities (164), and specific antisera to IFN-gamma, but not to IFN-α/β, neutralized all of the MAF activity produced by Schreiber's hybridoma (132). A confirmatory finding was that murine IFN-gamma produced by recombinant DNA technology (54) was active as a priming agent for tumoricidal activation, and the ratio of priming to antiviral activities of the

recombinant IFN-gamma was identical to that observed with the hybridoma-derived MAF (132,133). In both of these studies, coincidental sensitivities to pH and temperature were demonstrated, along with similar chromatographic properties on polynucleotide columns and Matrex Gel Red A (160). The identity of IFN-gamma as a MAF has been supported by others in similar experiments (104, 167,191).

While the preponderance of evidence indicates that IFN-gamma is the MAF that most laboratories have characterized, there exist a few examples of MAF's that are not IFN-gamma. The lymphokine originally described as macrophage cytotoxicity factor (MCF), derived from Con A-stimulated C57BL/6 spleen cells, was reported to be unadsorbed by poly(I)-Sepharose and thereby separated from interferon activity (84). T-cell hybridoma-derived MAF production was reported by Ratliff et al. (145) and Erickson et al. (45) in which no concomitant production of IFN-gamma activity was evident. The former hybridoma became unstable and was lost. Therefore, no further characterization of this MAF was possible. The latter study utilized a constitutive producer of MAF; all other hybridomas have required stimulation by a lectin for production. Finally, supernatants from the thymoma EL-4, stimulated with phorbol myristate acetate, were found to contain heterogeneous macrophage activating activities, 50-75% of which could be neutralized with anti-IFN-gamma antibodies, but a distinct

activity that could not be neutralized. The latter activity had an estimated molecular weight of 23,000 daltons and was acid-stable (pH 2, 18 hr) (109). The 23,000-dalton EL-4-derived MAF has recently been identified as B cell stimulatory factor-1 (BSF-1) (34). Another T cell-derived lymphokine that has been shown to activate human monocytes for tumoricidal function is human granulocyte-macrophage colony-stimulating factor (GM-CSF) (53). GM-CSF is also active in the mouse system (34). In this study, it was also shown that combination of BSF-1 and GM-CSF enhance the tumoricidal activity of murine macrophages over that induced by BSF-1 alone (34).

While the T cell-derived factors are important for macrophage activation, it has been well-established that type I interferons (α and β) are also effective for priming macrophages (19,131). The induction of α/β interferons by many other substances offers an explanation for the phenomenon of "direct" activation by many of these stimuli, including lipopolysaccharide and other polyanions such as polyinosinic:polycytidylic acid (166,185). These stimuli induce α/β interferon production by the macrophage, which then primes in an autocrine manner, while the stimuli themselves provide the triggering signal. The role of α/β interferon in the regulation of macrophage actrivation is more complex, however. If macrophages are pretreated with α/β interferons, they lose their responsiveness to IFN-

gamma and LPS for the development of tumoricidal activity (129). The effects of α/β interferons are clearly dependent upon when macrophages are exposed to them, relative to additional signals for activation. When the α/β interferons are offered simultaneously with a triggering agent (e.g., LPS), activation occurs within 24 hours. If either α or β interferon is introduced as little as 6 hours prior to addition of IFN-gamma and LPS, the macrophage response to the latter stimuli is severely diminished. The concentration of either IFN-α or -β that suppresses this response is far lower than that which is required for priming (129).

Gamma interferon has been determined to regulate most of the functions of macrophages previously attributed to lymphokine regulation. It not only induces priming and activation for tumor cell killing, but acts to maintain cytolytic function in the presence of prostaglandin E_2, a down-regulating signal for activation (152,182). IFN-gamma activates macrophages for enhanced microbicidal activity (69,118,121), at least in part by enhancement of H_2O_2 production induced by other signals (121). Fc receptor expression and immune phagocytosis are up-regulated by IFN-gamma (48,55,136,194). Expression of major histocompatibility antigens is also increased by IFN-gamma. Both Class I (81,202) and Class II or Ia (11,78,81,119,177, 202,205) antigens are up-regulated; the latter directly

affects the antigen-presenting capacity of these macrophages (13,119,205). Finally, IFN-gamma reduces the proliferative capacity of macrophage precursors (23,83,135,138) and induces their differentiation into macrophages (64,135). Clearly, many aspects of macrophage function are under regulation by gamma interferon. Most of the alterations induced by IFN-gamma result in the overall enhancement of the host defense potential of these cells.

Regulation of Activation for Tumor Cell Killing by Gamma Interferon and Lipopolysaccharide: Cell Surface Interactions

Murine gamma interferon is a polypeptide that is 132 amino acids in length (54). The interaction of polypeptide hormones that are active at extremely low concentrations with their target cells indicates the requirement for their interaction with specific cell surface receptors. The earliest suggestion of the need for interaction of interferon with a cell surface structure was made by Friedman in 1967 (52), before the distinctions of interferon subtypes (α, β, gamma) were made (10). Branca and Baglioni were the first to distinguish receptors for Type I (α, β) and Type II (gamma) interferons by demonstrating competition for ^{125}I-labelled human IFN-α binding to lymphoblastoid cells by the former but not the latter (188). The first direct studies of receptors for gamma interferon were in 1982 by Anderson et al. (8,9).

Human gamma interferon, labelled with ^{125}I-Bolton-Hunter reagent, was used to characterize specific IFN-gamma binding sites on human GM-258 fibroblasts. Scatchard analysis (157) indicated the presence of 2400 binding sites per cell with a dissociation constant (Kd) of 1.5 x 10^{-10}M. The binding was inhibited by unlabelled IFN-gamma and partially inhibited by IFN-β, but not IFN-α. Neutralizing rabbit antibodies to IFN-gamma inhibited interferon binding to cells (8). No report since that time has found competition for IFN-gamma binding by IFN-β. The results of Anderson et al. (8) may have been caused by the use of an impure preparation of naturally-derived IFN-gamma or IFN-β. When human IFN-gamma was immunoaffinity-purified to homogeneity and used to characterize IFN-gamma binding to WISH amnion cells and FSII fibroblasts, no competition for IFN-gamma was demonstrated with either α or β interferons that had been similarly purified (127). In this study, IFN-gamma bound to FSII cells with a Kd of 1 x 10^{-12}M (5,500 sites/cell) and to WISH cells with a Kd of 2.6 x 10^{-12} M (19,500 sites/cell). The specificity of α/β versus gamma interferon receptors was later verified using recombinant-derived forms of all three ligands (111).

Interaction of ^{125}I-IFN-gamma with GM-258 fibroblasts was further characterized as to internalization and degradation of the ligand (9). Labelled IFN-gamma was rapidly internalized at 37°C with a $t_{½}$ of 4-5 minutes and

subsequently degraded. Over eight hours, the uptake of IFN-gamma exceeded the number of occupied receptors by five times. As the ligand uptake was only moderately affected by cycloheximide, it was concluded that receptors were recycled. Degradation was inhibited by chloroquine, suggesting that it took place in lysosomes (9). Recent work has confirmed that murine IFN-gamma is internalized and degraded by mouse macrophages at 37° C. Degradation occurred at a constant rate (7000 molecules/cell/hour) and was inhibited by lysosomotropic amines. The receptor was continuously recycled, due to the presence of a substantial intracellular pool of receptors (29).

Early evidence for the presence of receptors for IFN-gamma on macrophages was presented by Celada et al. (27). IFN-gamma activity was absorbed by macrophages, even at 4° C, from a T cell hybridoma supernatant fluid. IFN-gamma coupled to fluorescent microspheres bound to macrophages; this binding was inhibited by an IFN-gamma-containing hybridoma supernate. Finally, ^{125}I-labelled recombinant-derived murine IFN-gamma bound to bone marrow macrophages in a specific and saturable manner with a Kd of 1.1 x 10^{-8} M. There were an estimated 12,000 binding sites per macrophage (27).

Accessibility to purified recombinant-derived interferons resulted in numerous reports of IFN-gamma receptor characterization in both human and mouse systems.

Receptors for recombinant human IFN-gamma were characterized on lymphoblastoid (Daudi) cells (98,111), HeLa cells (98), embryonic fibroblast lines (111), peripheral blood lymphocytes (111), numerous other human tumor lines (16,186,187), and monocytes and monocytic cell lines (26,50,128,143). Binding affinities ranged from 10^{-11}M in one laboratory (16,187) to 10^{-9}M (26,111). The number of receptors per cell varied widely, from undetectable to 20,000; one earlier study reported 50,000-70,000 sites per cell on WISH cells (156). With the exception of isolated human tumor cell lines, no cell type yet examined has failed to express receptors for gamma interferon (187). While the majority of receptor studies have been performed with ^{125}I-labelled interferons, a desire for greater sensitivity and higher specific activities led Rashidbaigi et al. to develop a new radio-ligand, IFN-gamma labelled with ^{32}P (143). Binding of ^{32}P-IFN-gamma to U937 histiocytic lymphoma cells yielded comparable results to ^{125}I-IFN-gamma binding to the same line (50). It was found in the ^{32}P-IFN-gamma study that IFN-gamma containing an additional three N-terminal amino acids (Cys-Tyr-Cys) was bound with 10-fold lower affinity than the natural IFN-gamma molecule (143). The former IFN-gamma was produced as a result of the predicted sequence from the cDNA, and has often been used as a radioligand for IFN-gamma receptor studies. The naturally-derived IFN-gamma was discovered to

lack these amino acids, and has a pyroglutamate at its amino-terminus (147). These differences may offer one explanation for the variations in affinity of IFN-gamma binding reported in the literature.

Detailed studies of gamma interferon receptors in the mouse were not reported until more recently (4,89,200). Weitzerbin et al. reported characterization of the IFN-gamma receptor on mouse L1210 leukemia cells, which expressed approximately 4,000 receptors per cell with a Kd of 5 x 10^{-10}M (200). Preparation of ^{32}P-labelled mouse gamma interferon and its binding to L cells, 258C4.4 (a cloned T cell line), and Nulli-SCC1 embryonal carcinoma cells was demonstrated by Langer et al. (89). ^{32}P-IFN-gamma bound with Kd from 10^{-9} to 10^{-10}M to these cells, and receptor numbers ranged from 5,000-15,000 per cell. Interestingly, the induction of differentiation of Nulli-SCC1 cells by retinoic acid resulted in a 2.5-5-fold increase in receptor number (89). However, the induction of differentiation of U937 and HL60 cells with phorbol myristate acetate or dimethylsulfoxide failed to affect IFN-gamma binding in a separate study (26).

An investigation of the binding characteristics of IFN-gamma to mouse macrophages led Aiyer et al. to postulate the existence of two classes of receptor on these cells, of high (Kd = 9.1 x 10^{-11}M) and low (Kd = 2.7 x

10^{-9}M) affinity (4). The affinity of the former site was more consistent with the concentrations of IFN-gamma required to elicit a functional response, in this case the induction of Ia and H-2 antigens; however, even at the proposed higher affinity, only about 5% of the receptors were occupied at the half-maximal response (4). An alternative explanation of these data, considered but not favored by the authors, is that endocytosis of the receptor-ligand complex, degradation of the ligand, and recycling of the receptor at 37°C (the temperature at which the binding experiments were performed) could result in the curvilinear nature of the Scatchard plots described in this report (201). Internalization of IFN-gamma has been demonstrated by several laboratories (9,26,200). In one of these studies, a curvilinear Scatchard plot indicating high (Kd = 4.3 x 10^{-11}M) and low (Kd = 5.9 x 10^{-10}M) affinity sites was derived at 37°C, but not at 4°C, on paraformaldehyde-fixed cells, or on isolated membrane preparations, suggesting that the data at 37°C were a result of internalization of the ligand (26). Degradation of IFN-gamma occurred rapidly at 37°C in human cells (9,26), but not in mouse L1210 cells, even though it was internalized (200). In view of the fact that normal membrane turnover in macrophages is quite extensive (179), it is not surprising that internalization of ligands by these cells affects binding parameters to such a significant degree.

The characterization of IFN-gamma receptors on macrophages was further complicated by data suggesting that properties of the binding site on cells of this lineage differed from those on nonhematopoietic cells (128). Treatment of IFN-gamma with formic acid, which resulted in 95% inactivation of its antiviral activity, yielded a ligand which competed for binding of ^{125}I-IFN-gamma to WISH amnion cells, but not to monocytes. The acid-treated IFN-gamma was able to induce HLA-DR antigen expression on WISH cells, but not on monocytes; untreated IFN-gamma induced HLA-DR antigens on both cell types, but only antiviral activity in the WISH cells. Finally, equilibrium saturation binding curves obtained on WISH cells yielded a linear Scatchard plot, while on monocytes, an upward concave dependency curve was obtained (128). While the last observation may be explained by the complications of internalization discussed above, the acid-treated IFN-gamma data suggests that two distinct types of IFN-gamma interaction may exist with monocytes in contrast to WISH cells.

The regulation of expression of receptors for IFN-gamma is poorly understood. Aside from the up-regulation of receptor number upon differentiation of Nulli-SCC1 cells (89), the only other known regulatory agent of receptor expression is the homologous ligand, IFN-gamma. Cells pre-treated with IFN-gamma were reduced in their capacity to

bind ^{125}I-IFN-gamma in both mouse (200) and human (98) cells. This effect was shown to be dose-dependent, specific for IFN-gamma (Type I IFN's had no effect), and reversible. The recovery of IFN-gamma binding after down-regulation was blocked by cycloheximide and tunicamycin, indicating the requirement for de novo synthesis of glycoproteins (200).

The active turnover of receptors for gamma interferon may provide an explanation for the general finding that physiologically active concentrations of IFN-gamma are quite low relative to the affinities of IFN-gamma binding, such that only a few receptors would be occupied at these concentrations. It has been suggested that there is an inverse relationship between the number of receptors expressed on the cell surface and the limiting concentrations of IFN-gamma required for the induction of HLA-DR antigens on various human tumor cell lines (16,186). The kinetics of response are an important factor. A minimal time of exposure to the ligand is apparently required, and the length of exposure necessary to elicit a response is inversely proportional to the concentrations of the ligand and receptor (16). The hypothesis that has been advanced is that the intracellular signal accumulates with repeated cycles of receptor-ligand internalization (16,29). It has been suggested that the number of cycles of receptor occupancy required for induction of functions depends upon

the function, e.g., enhanced H_2O_2 secretion requires only one round of receptor occupancy, while activation for tumor cell killing requires three to four rounds (29).

The nature of the intracellular signal(s) by which IFN-gamma induces the pleiotropic responses that it mediates is still unclear. However, some clues to the potential coupling events between binding of IFN-gamma and its ultimate effects have been revealed in pharmacological studies of IFN-gamma action. Using the down-regulation of transferrin receptors by IFN-gamma as a model, Weiel et al. (198) reported that the calcium ionophore A23187 and phorbol myristate acetate (PMA) each independently mimicked this effect of IFN-gamma. Phorbol esters known to activate protein kinase C were all similarly active in this system. Furthermore, suboptimal concentrations of PMA and A23187 cooperatively reduced transferrin receptor expression in macrophages. The results implied potential roles of elevated intracellular Ca^{++} and stimulation of protein kinase C in IFN-gamma action (198). The converse observation, stimulation of protein kinase C activity by treatment of macrophages with IFN-gamma, reinforced this hypothesis (60). Using bone marrow culture-derived macrophages, Johnson and Torres demonstrated directly that calcium ionophore A23187 could prime macrophages for tumor cell killing. PMA, diacylglycerol, arachidonic acid, leukotriene B_4, and platelet-activating factor were all

ineffective in this system (75). Somers et al. subsequently showed that PMA and A23187, in combination, were able to prime peritoneal macrophages for tumoricidal capacity. In addition, an intracellular Ca^{++} chelator (Quin-2/AM), but not an extracellular Ca^{++} chelator (EGTA), blocked priming of macrophages by IFN-gamma (173). These results were confirmed and extended by Celada and Schreiber; antagonists of calcium-binding proteins (of which protein kinase C is one) were found to block priming of macrophages by IFN-gamma (28). The A/J mouse provided a model for unresponsiveness to IFN-gamma for tumoricidal activation. Macrophages from these animals exhibit normal binding of IFN-gamma, and can be primed with A23187 and PMA, but do not show increases in protein kinase C activity or efflux of intracellular calcium in response to IFN-gamma (61). All of these results, taken together, indicate the potential importance of protein kinase C activity and particularly intracellular calcium levels in the regulation of IFN-gamma-mediated priming of macrophages for tumor cell killing. It is not yet known whether the IFN-gamma receptor is coupled to the phospholipase-dependent cleavage of phosphatidylinositol-4,5-bisphosphate into diacylycerol (which activates protein kinase C) and inositol-1,4,5-triphosphate (which increases intracellular Ca^{++} concentrations).

Complete understanding of the mechanisms involved in transduction of the IFN-gamma signal will require the biochemical characterization of the receptor and any associated cell surface molecules. The first attempts at biochemical characterization of the receptor for IFN-gamma involved the use of crosslinking techniques. Crosslinking of ^{125}I-labelled human IFN-gamma to WISH cells using either disuccinimidyl suberate (DSS) or ethylene glycol bis (succinimidyl succinate) yielded a labelled complex that migrated with an apparent molecular mass of 105,000 ± 5,000 daltons when analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Formation of the complex was inhibited by preincubation with unlabelled IFN-gamma, but not with unlabelled IFN-β (156). Human IFN-gamma that had been acylated with N-succinimidyl-6 (4'-azido-2'-nitrophenylamino) hexanoate, a photoreactive crosslinker, formed a complex upon binding to GM-258 fibroblasts and photoactivation that migrated with an Mr of 230,000 ± 7,000 daltons (7). Several other reports using crosslinking techniques indicated the formation of complexes of varied molecular weights. Using DSS, Littman et al. reported the formation of a complex of Mr = 125,000 ± 20,000 from Daudi and HeLa cells (98). Celada et al., crosslinking ^{125}I-IFN-gamma to U937 cells or membranes, demonstrated a diffusely migrating complex of 84,000-107,000 daltons (26). Murine IFN-gamma crosslinked to

L1210 cells yielded a complex of Mr = 110,000 ± 5,000 daltons (200). Using ^{32}P-IFN-gamma, Langer et al. reported the formation of a complex of Mr = 90,000-97,000 daltons from mouse cells (89) and 117,000 ± 17,000 daltons from human cells (144). The former estimate was later changed to 110,000 ± 15,000 daltons for the murine receptor complex (106). A heterogeneous pattern of complexes was reported by Ucer et al. (187) upon crosslinking of IFN-gamma to various human tumor lines. Three distinct bands of 70 ± 5, 92 ± 5, and 150 ± 10 kilodaltons were seen in all cells tested. In all of these studies, formation of the complexes was inhibited by prior incubation of the cells or membranes with unlabelled IFN-gamma. The complexes formed always migrated on SDS-PAGE to form a band of a diffuse nature.

Direct biochemical analysis of the receptor has so far been limited to the human system. Polyclonal antibodies to the human IFN-gamma receptor were prepared by immunizing rabbits with a receptor-rich cell line and extensively adsorbing the sera with a receptor-negative cell line. The resultant antibodies immunoprecipitated internally labelled molecules with a Mr of 75,000 to 90,000 by SDS-PAGE (87). The human IFN-gamma receptor has been purified by affinity chromatography on immobilized IFN-gamma columns (2,124, 125). Novick et al. reported the recovery of three molecular species, upon elution of Triton X-100 extracts

from an immobilized IFN-gamma column; a major band of Mr = 95,000 and two minor bands of Mr = 60,000 and 80,000 were revealed by SDS-PAGE under reducing conditions. Nonreducing conditions yielded a major band of Mr > 200,000 (124,125). Polyclonal antibodies produced in mice by immunization with the 95,000-dalton protein possessed neutralizing activity (antiviral) specific for IFN-gamma, and immunoprecipitated a ^{125}I-IFN-gamma-receptor cross-linked complex of Mr = 120,000 (125). Aguet and Merlin (2) reported purification of the human IFN-gamma receptor from Raji lymphoma cells by sequential affinity chromatography on an IFN-gamma column and a column to which a monoclonal antibody prepared to enriched receptor was coupled. The final eluate consisted of two molecular species, of Mr = 90,000 and 50,000. Both of these proteins bound IFN-gamma on nitrocellulose blots. The two proteins migrated to the same extent under both reducing and nonreducing conditions. The relationship between the two is unclear. The 50,000-dalton protein may represent a degradation product of the 90,000-dalton molecule, or the two species may be indicative of receptor subunits. The definitive structure of the IFN-gamma receptor awaits further characterization, particularly with regard to putative subunits and/or associated components that may be important for transduction of the IFN-gamma signal. It will be of particular interest to see if there are structural

differences between receptors on macrophages and non-hematopoietic cells, as hypothesized by Orchansky et al. (125,128). This hypothesis was based primarily on differences in the ability of acid-treated IFN-gamma to induce HLA-DR antigens and antiviral activity on WISH cells and monocytes (128). Intact IFN-gamma appeared to be required for antiviral activity on WISH cells and induction of HLA-DR on monocytes. It would be of interest to determine if other, more complex functions such as microbicidal or tumoricidal activity by macrophages were also dependent upon intact IFN-gamma. Acid-treated IFN-gamma was able to induce HLA-DR on WISH cells, indicating that both cell type and function induced were distinguishable by this procedure. One possibility that has precedent is that the receptor is the same on all cell types, but contains distinct cytoplasmic domains that may be associated with different functional signals, as with the Fc receptor (146). The availability of antibodies to the receptor (2,125) should prove invaluable in delineating important structural characteristics required for IFN-gamma action.

The absolute requirement of IFN-gamma receptor engagement for macrophage activation and antiviral activity has been called into question by two different sets of experiments. First, encapsulation of IFN-gamma into liposomes created a vehicle for IFN-gamma action that

appeared to bypass interaction with the receptor, since it was refractory to neutralization by antibodies to IFN-gamma and resulted in the abrogation of species (human-mouse) specificity of the ligand (49). Although questions of liposome leakage made these data controversial (44), the ability to bypass the need for extracellular receptor interaction was reinforced by transformation experiments, in which mouse cells transformed with a human cDNA encoding a non-secreted form of IFN-gamma acquired permanent antiviral activity (155). These cells were transfected with a cDNA that lacked the signal sequence required for secretion of the ligand; these cells accumulated the IFN-gamma intracellularly, where it could not interact with the cell surface receptor. These experiments also suggested that species specificity of IFN-gamma is maintained at the receptor level.

The nature of the interaction of triggering signals for tumoricidal activation of macrophages with these cells is much less clear, with the possible exception of the demonstration of receptors for maleylated proteins that appear to mediate the secretion of neutral proteases by macrophages (74). The most commonly utilized and well-studied triggering agent for macrophage activation is bacterial lipopolysaccharide (LPS) (68,108,130,150). The existence of a specific "LPS receptor" that might mediate the triggering signal has been the subject of controversy

(reviewed in 117). Springer et al. (176) were the first to describe the presence of an LPS receptor on erythrocytes; it was characterized by virtue of its ability, once isolated, to displace ^{32}P-labelled LPS from erythrocytes. The receptor was isolated and purified to apparent homogeneity; it was identified as a 256,000-dalton lipoglycoprotein rich in N-acetyl-neuraminic acid, galactose, and hexosamines (175). Attempts by this group to characterize a similar receptor on leukocytes and platelets failed. Instead, LPS-binding activity from these cells was found to be of low specificity (binding other bacterial antigens) and composed entirely of lipid (174).

The activity of LPS as a triggering agent, as well as for most other functions, is entirely attributable to the lipid A component (41). The availability of inbred strains of mice that are hyporesponsive to the numerous effects of LPS and lipid A provided a powerful tool for dissecting the interactions of LPS and macrophages that lead to a response (110). Taking advantage of such a system, Couthino et al. prepared an antiserum reactive to the "lipid A-specific triggering receptor" on B cells by immunizing rabbits with C3H/Tif (LPS high responder) cells and extensively absorbing the resulting serum on C3H-HeJ (LPS low responder) cells. Lipopolysaccharide and lipid A competed for the binding of this antiserum to C3H/TifB cells, and the anti-

body was mitogenic for these cells (32). Unfortunately, these studies have not been reproducible (197).

Several attempts to study the binding of LPS or LPS components directly to cells have been made in recent years. Davies et al. described the binding of ^{32}P- and ^{14}C-labelled LPS to various cell types and demonstrated an unusual cyclical absorption-desorption pattern of binding with a periodicity of around 60 minutes (38). A specific LPS receptor on rabbit peritoneal macrophages was characterized using ^{3}H-LPS as a ligand. In this study ^{3}H-LPS was found to bind reversibly and specifically to both resident and elicited peritoneal macrophages, but not to lung macrophages (to which LPS bound irreversibly and nonspecifically). It was determined that the macrophages bound 3×10^4 LPS molecules per cell, and ligand specificity was assessed by lack of competition with LPS from a distinct bacterial species. The binding was apparently mediated through the polysaccharide component of the LPS, and it was not inhibited by the lipid A component (58). More recent studies of this binding phenomenon indicated a requirement for the presence of serum for binding to occur, and implicated a potential role for the complement component C3. Specific binding of ^{3}H-LPS was demonstrated for both human monocytes and mouse macrophages in this system (56). As the majority of effects of LPS are mediated via the lipid A region, the existence of a

polysaccharide-specific receptor may be of minimal functional importance. However, it has been shown that the isolated polysaccharide moiety of LPS from Bordetella pertussis can activate B cells in a macrophage-dependent system, and that this apparently occurs via the stimulation of IL-1 secretion from the macrophages (57). The interactions of ^{3}H-LPS and ^{51}Cr-labelled lipid A with human monocytes have also been studied. ^{3}H-LPS bound rapidly ($t_{½}$ < 5 minutes) and reversibly to monocytes, followed by a gradual loss in binding over time, if the assay was terminated using trichloroacetic acid. However, if the assay was terminated by cold filtration, a more gradual, irreversible binding was noted. Use of ^{51}Cr-lipid A as a ligand resulted in only the former type of binding. If monocytes were pretreated with LPS, the number of binding sites (but not the affinity) diminished (92). When membranes were prepared from monocytes, binding of ^{3}H-LPS and ^{51}Cr-lipid A was less specific and of lower affinity than to whole cells, leading the authors to conclude that some assembly of structures in the lipid bilayer of the intact cell membrane could occur that were required for the efficient high affinity specific binding of LPS and lipid A. The speculation was that this assembly could not occur after membranes were prepared from the cells (91). Other studies with radiolabelled LPS have yielded results that were not indicative of a specific receptor. Warner et al.

demonstrated binding of ^{125}I-LPS to human monocytes that was neither saturable with time nor LPS concentration, and that was inhibitable by the O-polysaccharide but not by lipid A or polymyxin B (an antibiotic that binds to lipid A) (196). The preponderance of evidence related to binding of LPS to cells has indicated that binding is probably necessary, but not sufficient, for the induction of its effects (117). One of the overriding complications of studying the interaction of LPS with cell membranes is the high degree of nonspecific interactions associated with LPS. LPS interacts strongly with a number of membrane components such as glycoproteins, glycosphingolipids, and phospholipids (115,117). Recently, the potential importance of gangliosides in LPS responses has been reported (31,153). The interaction of LPS with monocyte and macrophages has been shown to result in an increase in plasma membrane microviscosity and a decrease in the order of the lipid bilayer (90). There is a transient perturbation in the membranes of primed (therefore hyperresponsive to LPS) but not elicited macrophages upon exposure to LPS (46), suggesting a role for this perturbation in the triggering response for tumor cell killing. The tendency of LPS to interact with plasma membrane components in an avid manner may be of primary importance to the ultimate generation of the signals leading to the numerous responses of cells to this

molecule. Morrison and Rudbach (117) have proposed that both intercalation of LPS in the lipid bilayer and interaction with high affinity receptors are required for functional responses to LPS. In this hypothesis, the lipid bilayer interaction would precede the receptor binding. Consistent with this model is that LPS interactions with membranes and responses to LPS are time- and temperature-dependent, and they are dependent upon the phospholipid composition of the membrane bilayers. Finally, these authors maintain that LPS-induced membrane perturbations may be sufficient for some functional responses that require a second signal (e.g., Con A or PMA), but not for other responses (117). Although these are a variety of membrane components with which LPS may interact strongly enough to induce such perturbations, there is good evidence for the role of gangliosides in the response of macrophages to LPS, based on their ability to inhibit LPS-induced functions in macrophages (31,153).

One attractive hypothesis for the interaction of LPS with the plasma membrane and the coupling of this interaction to responses is that LPS inserts itself into the bilayer and subsequently associates specifically with some membrane component(s) that is(are) responsible for the genesis of a transmembrane signal. Intriguing evidence for this hypothesis was reported by Kilpatrick-Smith et al., who performed photobleaching experiments using LPS

conjugated to fluorescein isothiocyanate (80). These studies allowed the determination of the lateral mobility of bilayer-associated LPS. It was observed that there were two subpopulations of LPS bound to the cell, one of rapid mobility ($D = 10^{-9}-10^{-8}$ cm^2/s) and one of relative immobility ($D < 10^{-12}$ cm^2/s) (D = lateral diffusion coefficient). These results suggested to the authors that at least some LPS, after insertion into the plasma membrane, became associated with an anchored membrane component (80).

The interaction of LPS with the cell surface that leads to a stimulus and response is still poorly defined. Recent evidence suggests that LPS may induce signal transduction via the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), which would lead to increased intracellular calcium levels (mediated by inositol-1,4,5-triphosphate) and activation of protein kinase C (mediated by diacylglycerol). PIP_2 hydrolysis occurred with levels of LPS that were physiological relevant to macrophage activation (139). There is also evidence to implicate a role for guanine nucleotide regulatory proteins in the mediation of LPS responses by B cells and macrophages (72). Earlier studies have suggested a possible role for activation of cell-associated serine proteases in generation of LPS-induced responses. Several laboratories reported mimicry of LPS stimulation of B cells by trypsin

and inhibition of LPS stimulation by serine protease inhibitors such as soybean trypsin inhibitor, N-α-tosyl-L-lysyl-L-chloromethane (TLCK) and di-isopropyl phosphofluoridate (DFP) (12,62,63,76,114). While endotoxin contamination in trypsin preparations calls the former observation into question, the latter results suggested that mediation of LPS responses (by B cells) occurred via the early action of a cellular serine protease. In support of this hypothesis is the fact that LPS activates at least two soluble serine proteases, factor XII of the intrinsic coagulation system and the complement component C1 (115).

Characterization of a specific receptor for LPS that is responsible for initiating a response should help to clarify the means by which LPS induces its many effects. A series of experiments reported by Wright and Jong (204) implicate the family of adhesion-promoting receptors as LPS receptors. Specifically, antibodies to the 95,000-dalton B chain common to CR3 (receptor for the complement cleavage product C3bi), lymphocyte function-associated antigen (LFA-1), and p150,95 (a dimeric protein of unknown function) removed the capacity of human macrophages to bind LPS (as E. coli or LPS-coated erythrocytes). This binding was time- and temperature-dependent, and mediated by lipid A. The authors speculated that the receptor recognized a common structure, such as a sugar phosphate, on the lipid A portion of the LPS (204).

Summary and Conclusion

The important role of macrophages in host defense has been well documented. These cells are unique in the versatility of their functional activities, ranging from the phagocytosis of a variety of particles, chemotaxis, and participation in the immune response, to their microbicidal and tumoricidal functions. Virtually all of the activities of macrophages are induced or regulated by environmental stimuli. The concept of macrophage activation has been the subject of intense investigation since its original description by Mackaness (101,102). Although the phenomenon of macrophage activation for tumor cell killing has been well described (1), the sub-cellular and molecular events involved in the induction of activation are poorly understood. Characterization of these molecular interactions is critical to the ultimate understanding of how macrophage tumoricidal activities are regulated.

Significant progress in the delineation of the molecular events involved in macrophage activation was made possible by the identification of the stimuli required for the induction of the priming and triggering stages of activation. Of central importance was the cloning and purification of gamma interferon, a lymphokine with potent priming activity on macrophages. Many triggering stimuli have also been identified, such as LPS, heat-killed

Listeria monocytogenes, double-stranded RNA, maleylated and acetylated proteins, muramyl dipeptide, and lymphokines.

The interaction of many activating stimuli with macrophages is poorly understood. Many laboratories have reported binding parameters for gamma interferon on various cell types, but only two groups have reported studies using murine macrophages, with highly disparate results (4,27). Studies on murine macrophages are critical due to the fact that these cells have been extensively used to rigorously characterize the phenomenon of activation for tumor cell killing. The nature of the interaction of triggering stimuli with macrophages is largely unknown. Specific receptors have been characterized for maleylated proteins (74), and extensive efforts to identify receptors for LPS have been undertaken (117), but triggering of primed macrophages for tumoricidal activation remains poorly understood at the subcellular level.

The studies to be described here examine the interactions of various agents involved in macrophage activation with murine macrophages, either directly (in the case of MuIFN-gamma interaction) or indirectly (in the case of triggering stimuli), and finally describe the first steps toward isolation and purification of the MuIFN-gamma receptor for further molecular studies. These results provide insights into the cell surface interactions involved in the induction of macrophage activation, and

provide the groundwork for the purification of a specific receptor for murine gamma interferon. The latter goal will permit the production of antibodies to the receptor and its molecular characterization, which represents the next vertical step in the understanding of the induction of macrophage activation for tumor cell killing.

CHAPTER II

THE INTERACTION OF MURINE GAMMA INTERFERON WITH MACROPHAGES

Introduction

Gamma interferon has multiple effects on macrophages which, taken together, result in an overall enhancement of their immune and host defense functions. These include the up-regulation of Fc receptors and histocompatibility antigens, the enhancement of microbicidal activity, and the induction of nonspecific tumoricidal activity (reviewed in 161,192,203). In most instances, gamma interferon does not fully activate macrophages for tumor cell killing, but rather primes them to respond to a second, triggering signal, most commonly provided by bacterial lipopolysaccharide (LPS) (132). Macrophages can be activated with gamma interferon alone under certain conditions; this phenomenon is particularly dependent upon the strain of mouse used for study (134). Therefore, the physiological relevance of priming and triggering remains controversial.

The first step toward understanding the mechanism(s) by which gamma interferon mediates its pleiotropic effects is the characterization of a cell surface receptor for this

lymphokine. Specific, high affinity receptors for gamma interferon have been demonstrated on numerous cell types of both human (8,16,26,50,98,111,128,143,156,186,187) and mouse (4,27,89,200) origin. The majority of studies on IFN-gamma interactions with macrophages or macrophage-like cell lines have been with human cells (26,50,128,143). Receptors for IFN-gamma on mouse macrophages have been described, although there is little agreement on the binding parameters derived in the two extant studies (4,27). The substantial inconsistencies in these data necessitated the studies described here, under conditions which the significant endocytic activity of macrophages were known to be minimized. Murine gamma interferon was labelled to high specific activity with ^{125}I and used to characterize the interaction of IFN-gamma with various mouse macrophage populations. The results indicated the presence of a single class of specific, high affinity binding sites for IFN-gamma on macrophages. Populations of macrophages varying in their responsiveness to IFN-gamma for activation did not appear to differ significantly in receptor expression, suggesting that regulation of response to activating signals occurs at a level distinct from receptor interaction with the priming ligand.

Materials and Methods

Media and reagents. All experiments were performed in HEPES-buffered, modified Eagle's medium (HMEM). HMEM consisted of modified Eagle's minimum essential medium (Auto-Pow MEM) prepared with 2 mg/ml sodium bicarbonate, 2 mM glutamine (all from Flow Laboratories, McLean, VA), 100 U/ml penicillin G potassium, 100 µg/ml streptomycin (both from Pfizer, Inc., New York, NY), and 15 mM N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES; Sigma Chemical Co., St. Louis, MO). For experiments, HMEM was supplemented with 10% v/v fetal bovine serum (FBS; Sterile Systems, Inc., Logan, UT). Heparin sodium (O'Neal, Jones, and Feldman, St. Louis, MO) was used at 2 U/ml in HMEM for bone marrow and peritoneal harvest. For bone marrow culture, HMEM was supplemented with 15% v/v L cell-conditioned medium, 10% v/v FBS, and 5% v/v horse serum (Flow Laboratories, McLean, VA). L cell-conditioned medium was obtained from cultures of NCTC L929 cells grown at confluency for 4 days in HMEM/10% FBS, and filtered (0.45 µm) before use). All media tested negative for the presence of endotoxin by the Limulus amebocyte lysate (LAL) (Associates of Cape Cod, Inc., Woods Hole, MA) assay (97) at a sensitivity of 0.07 ng/ml. Purified, lipid A-rich fraction II of phenol-extracted bacterial lipopolysaccharide (LPS) from Escherichia coli 0111:B4 (116) was a gift from Dr. D. C. Morrison, Department of

Microbiology, University of Kansas School of Medicine, Kansas City, KS.

Bone marrow culture. Macrophages were obtained from bone marrow cultured as described by Leung et al. (96) as modified from Meerpohl et al. (107). Bone marrow was harvested from the tibiae and femora of 6-9 week old C_3H/HeN mice (Charles River Laboratories, Kingston, NY) and cultured in bacterial grade 100 mm^2 Petri dishes (Lab-Tek Products, Naperville, IL) at 37°C, in an atmosphere containing 5% CO_2. Bone marrow was seeded at 4 x 10^6 nucleated cells/dish in 15 ml of bone marrow culture medium. On day 7, 5 ml of the same medium were added to each dish. Cells were harvested for use between days 11 to 13 of culture using ice cold phosphate-buffered saline (PBS). Differential analysis of cytocentrifuge preparations stained with Diff-Quik (Harleco, Gibbstown, NJ) showed that populations of cells derived in this manner consistently contained 97-99% macrophages.

Peritoneal macrophages. Peritoneal exudate cells were harvested by lavage with HMEM containing 2 U/ml heparin. For inflammatory exudates, mice were injected intraperitoneally with 1.5 ml 10% protease peptone broth three days prior to harvest (130). Cells were incubated on tissue culture-grade 96-well plates (200,000/well) for 2 hours at 37°C for adherence, and nonadherent cells removed by washing twice before initiating assays.

Interferon. Mouse gamma interferon (MuIFN-gamma) was produced by E. coli into which an expression vector had been introduced that contained the MuIFN-gamma coding sequence (54,93). This reagent was a gift from Genentech, Inc. (South San Francisco, CA) (lot #1551/43) and Schering-Plough Pharmaceuticals (Bloomfield, NJ) (lots 18501-131 PF28-33 and 6-M6-01). The specific activity of the Genentech interferon was 5 x 10^6 U/ml and of the Schering interferons, 2 x 10^6 U/ml, as measured by the ability of each preparation to protect L929 cells against plaque formation by vesicular stomatitis virus (17).

Radioiodination of MuIFN-gamma. Gamma interferon was radiolabelled with ^{125}I using chloramine T (71). Six µg of interferon were diluted in 10 µl with 0.5 M sodium phosphate buffer, pH 7.5, and combined with 3-5 µl (300-500 µCi) of carrier-free Na ^{125}I (15 mCi/µg; Amersham Corp., Arlington Heights, IL) and 10 µl 5 mg/ml chloramine T (Sigma) for 60 seconds at 25°C. The reaction was stopped by the addition of 10 µl 10 mg/ml sodium metabisulfite and 10 µl 70 mg/ml potassium iodide. After adding 10 µl 20 mg/ml bovine serum albumin (BSA) and NaCl (4 M) to 1 M final concentration, the mixture was desalted on a 5 ml column of Bio-Gel P6-DG (Bio-Rad, Richmond, CA) that had been equilibrated with 10 mM Tris-HCl, pH 7.5, 1 M NaCl, containing 2 mg/ml BSA. The labelled preparations were stored at 4°C following addition of 2-mercaptoethanol to a

final concentration of 0.1% (v/v). Preparations were tested for biological activity by measuring the ability to prime macrophages for tumor cell killing (see below).

Assay for macrophage priming and activation. Priming was defined as acquisition by macrophages of the capacity to respond to a second, triggering signal (in this system, LPS) by developing cytolytic activity for tumor cells. To quantify priming activity (130), monolayers of bone marrow-derived macrophages (5 x 10^4/well in 96-well microtiter plates) were incubated at 37°C with different concentrations of MuIFN-gamma in the absence or presence of 1 ng/ml LPS, for the times indicated in the text. After the incubation period, monolayers were washed with HMEM/10% FBS. ^{51}Cr-labelled ($Na_2{}^{51}CrO_4$, 300 mCi/mg; Amersham Corp., Arlington Heights, IL) P815 mastocytoma cells were then added in 200 µl HMEM/10% FBS as targets (2 x 10^4/well). After 16 hours at 37°C, the upper 100 µl of supernate were removed and analyzed in an automatic gamma spectrometer to determine the extent of ^{51}Cr release. Cytotoxicity was expressed as % specific ^{51}Cr release as calculated using the formula:

$$\frac{\text{experimental cpm-spontaneous release cpm}}{\text{total releasable cpm-spontaneous release cpm}} \times 100.$$

Total releasable cpm was determined after freeze-thawing under hypotonic (50% distilled water) conditions. Spontaneous release was that which occurred when target cells

were held throughout the incubation period on unstimulated monolayers of macrophages.

Assay for binding of ^{125}I-MuIFN-gamma. Macrophage monolayers were prepared exactly as described above. After allowing cells to adhere, they were washed and exposed to 50 µl of 100 mM NaN_3 in HMEM/10% FBS for 15 minutes. In pilot studies, macrophages that were exposed to 100 mM NaN_3 for 15 minutes and then maintained in 50 mM NaN_3 stopped all detectable nonspecific endocytosis (using horseradish peroxidase as a marker). The cells were not irreversibly damaged, as full endocytic activity was recovered in NaN_3-free medium. An equal volume containing ^{125}I-MuIFN-gamma was next added to wells in the presence or absence of a 100-fold excess of unlabelled MuIFN-gamma. This concentration of unlabelled MuIFN-gamma inhibited binding by > 90%. Incubation at 37°C for 2 hours was followed by three rapid washes in ice-cold HMEM/10% FBS. Cells were then solubilized in 2% NP-40, harvested, and lysates were analyzed in an automatic gamma spectrometer. In some experiments, cells were fixed with 0.1% paraformaldehyde in phosphate-buffered saline to stop endocytic activity prior to the binding assay. In these cases, NaN_3 was omitted from the assay.

Results

Radioiodination of murine gamma interferon. Purified, recombinant-derived murine gamma interferon was radiolabelled with ^{125}I using chloramine T. Specific activities ranged from 10-60 µCi per µg protein. Greater than 50% of the original biological activity was retained in these preparations when tested for priming activity for tumoricidal function. Alternative labelling techniques resulted in lower specific activities with no significant improvement in recovery of biological activity. Desalting of unlabelled IFN-gamma indicated that significant losses of activity occurred on the column. These losses were overcome by performing the desalting procedure in the presence of 1 M NaCl. Figure 2-1 represents an autoradiograph of ^{125}I-MuIFN-gamma analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Two molecular species are evident: a major band migrating with an apparent molecular mass (Mr) of 16,000 daltons, and a less predominant band of Mr 32,000 daltons. The relative proportions of these species were unaffected under reducing versus nonreducing conditions. For the purposes of calculating binding parameters, it will be assumed that the monomer species (Mr = 16,000) was bound.

Establishment of conditions to control macrophage endocytic activity. Prior to attempting binding experiments with ^{125}I-MuIFN-gamma, it was necessary to

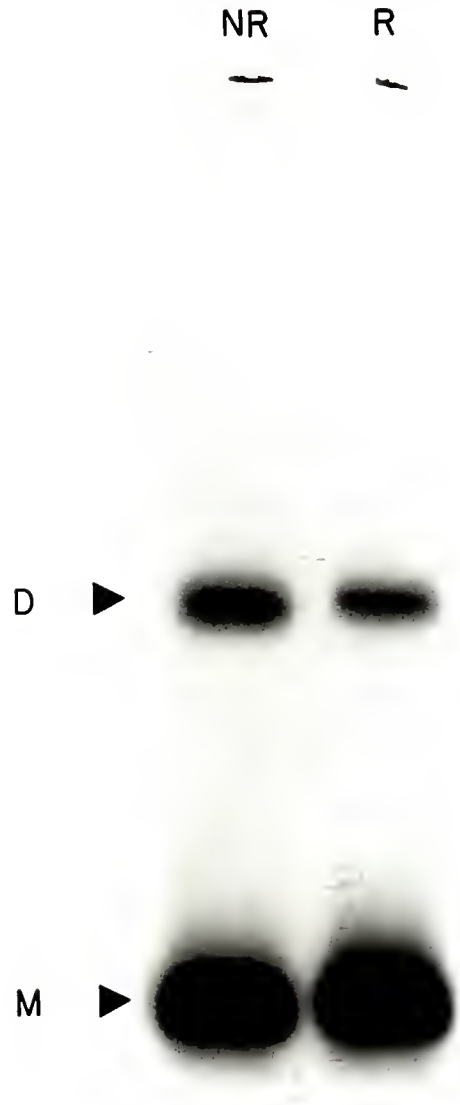

Figure 2-1. SDS-PAGE of ^{125}I-MuIFN-gamma.

^{125}I-MuIFN-gamma (100,000 CPM/lane) was subjected to electrophoresis on a 15% polyacrylamide resolving gel under reducing (R) or non-reducing (NR) conditions. Gels were stained with Coomassie blue for localization of molecular weight markers, and dried under heat and vacuum. Autoradiography using Kodak XAR-5 film at -70°C revealed two species of approximately 16,000 (monomer; M) and 32,000 (dimer; D) daltons.

determine conditions under which endocytic function, and therefore internalization of the ligand, could be controlled. Using horseradish peroxidase (HRP) as a marker of fluid-phase endocytosis, it was found that, at 37° C, high concentrations of sodium azide (NaN_3) were required to completely inhibit HRP uptake by bone marrow-derived macrophages. Such NaN_3 concentrations (up to 50 mM) were not irreversibly toxic to the macrophages, as removal of the NaN_3 resulted in a rapid recovery of endocytic function to control rates (data not shown).

Internalization of ^{125}I-MuIFN-gamma was directly estimated by acid-stripping experiments. Bone marrow-derived macrophages were exposed to ^{125}I-MuIFN-gamma in the presence or absence of a 100-fold excess of unlabelled IFN-gamma to determine specific binding to these cells under various conditions. At the end of 2 hours at 37° C, the cells were washed and treated with 0.2 M acetic acid, pH 2.5, containing 0.5 M NaCl for 5 minutes at 4° C to remove bound IFN-gamma remaining at the cell surface (59). The cells were then lysed with 2% NP-40 to harvest remaining (internalized or acid-resistant) counts. Table 2-1 indicates that under normal conditions, approximately two-thirds of the bound IFN-gamma was internalized or resistant to stripping. However, if the cells were treated with NaN_3 before and during the binding assay, or if they were fixed

Table 2-1. Inhibition of Internalization of ^{125}I-MuIFN-gamma by Macrophages

Treatment	% Acid-Sensitive CPM[d]	% Acid-Resistant CPM
medium alone[a]	33.9	66.1
PF[b]	80.2	19.8
NaN_3[c]	77.9	22.1

[a] Bone marrow culture-derived macrophages were allowed to adhere to tissue culture wells for 2 hrs at 37° C, washed, and exposed to 100 ng/ml ^{125}I-MuIFN-gamma ± 10 µg/ml unlabelled IFN-gamma for 2 hrs at 37° C. All incubations were in HMEM/10% FBS.

[b] Immediately prior to addition of ^{125}I-MuIFN-gamma, macrophages were fixed with 0.1% paraformaldehyde (PF) in phosphate-buffer saline for 5 min.

[c] Fifteen minutes before addition of ^{125}I-MuIFN-gamma, cells were exposed to sodium azide (NaN_3) in HMEM/10% FBS at a concentration of 50 mM; the presence of NaN_3 was maintained throughout the experiment.

[d] To remove surface-bound (but not internalized) CPM, macrophages were exposed to 0.2 M acetic acid, pH 2.5 containing 0.5 M NaCl for 5 min. at 4° C, and washed once with the same solution. Remaining (resistant) CPM were recovered by solubilization of the cells with 2% NP-40.

with 0.1% paraformaldehyde, only 20% of the bound ligand was acid-resistant. The complete lack of HRP internalization with NaN_3, coupled with the assumption that paraformaldehyde-fixed cells are not endocytically active, indicated that internalization of ^{125}I-MuIFN-gamma was inhibited to the greatest possible extent under these conditions.

Binding parameters of ^{125}I-MuIFN-gamma to macrophages. Under conditions designed to minimize endocytosis of the ligand, macrophages were incubated with ^{125}I-MuIFN-gamma (Genentech) to determine binding parameters for this ligand. Time course experiments at 37°C indicated that binding equilibrium was gradually achieved by 2 hours of incubation. Nonspecific binding, i.e., that which bound in the presence of a 100-fold excess of unlabelled MuIFN-gamma, remained unchanged over the same time, and was less than 10% of the total MuIFN-gamma bound by the cells (Figure 2-2). All subsequent binding experiments were performed for 2 hours at 37°C. The specificity of ^{125}I-IFN-gamma binding was further ascertained by incubating the radioligand with macrophages in the presence of increasing concentrations of unlabelled MuIFN-α, -β, or -gamma. Only the MuIFN-gamma competed effectively for binding of ^{125}I-MuIFN-gamma, while the Type I IFN's (α and β) were ineffective, even at concentrations up to 2500 U/ml (Figure 2-3).

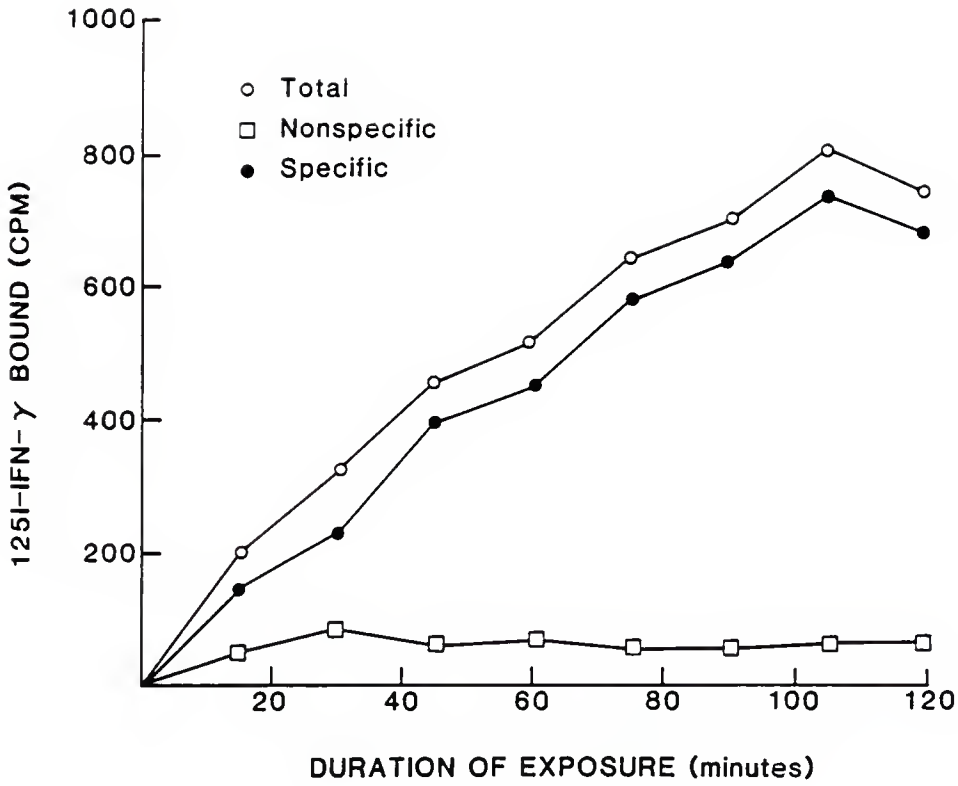

Figure 2.2. Time course of binding of ^{125}I-MuIFN-gamma to macrophages.

Bone marrow culture-derived macrophages were allowed to adhere to tissue culture wells for 2 hr, exposed to NaN_3 in HMEM/10% FBS as described in Materials and Methods, and then exposed to 10 U/ml ^{125}I-MuIFN-gamma for the times indicated on the abscissa at 37°C. At those times, monolayers were washed rapidly with three ice-cold volumes of HMEM/10% FBS and solubilized with 2% (w/v) NP-40. Nonspecific binding (that which bound in the presence of a 100-fold excess of unlabelled MuIFN-gamma) was subtracted from total binding (that which bound in the absence of unlabelled MuIFN-gamma) to calculate specific binding.

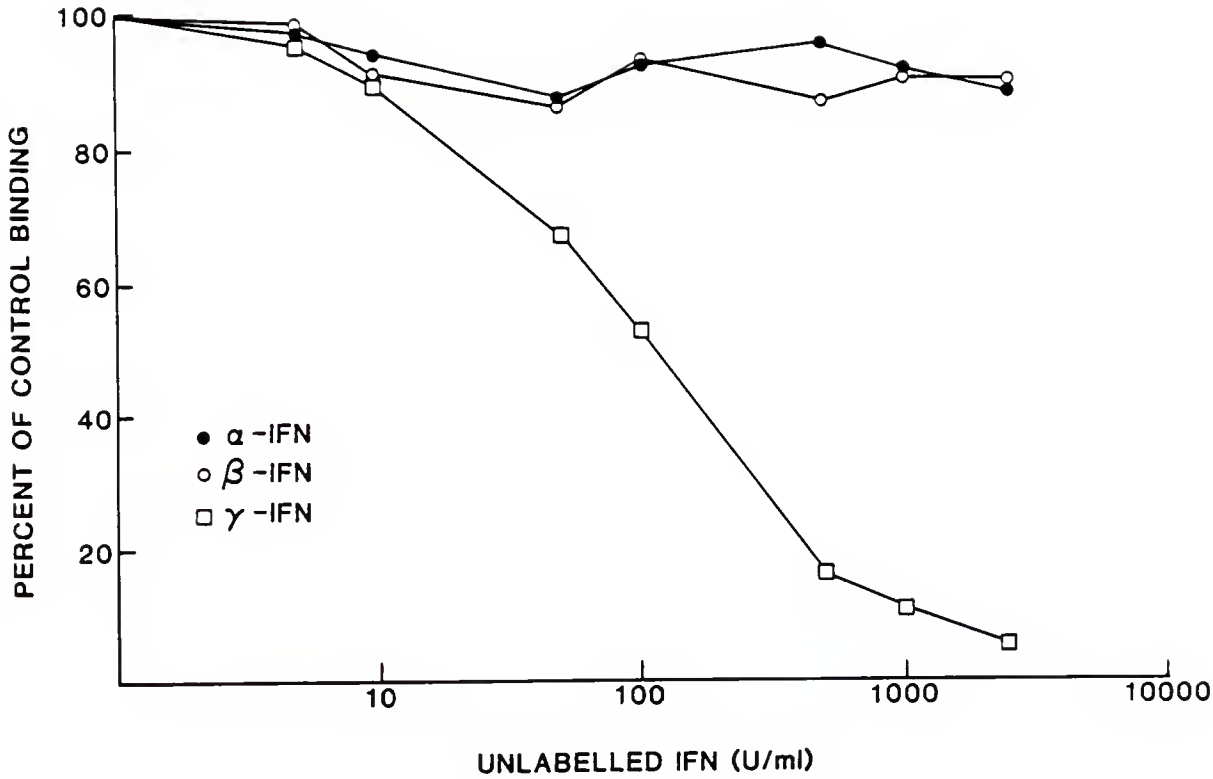

Figure 2-3. Specificity of binding of ^{125}I-MuIFN-gamma to macrophages.

Bone marrow culture-derived macrophages were adhered and treated with NaN_3 as in Figure 2-2. Cells were then exposed to a constant concentration of ^{125}I-MuIFN-gamma (50 U/ml) in the presence of the indicated concentrations of α-, β-, or gamma-interferons for 2 hr at 37°C. Binding was determined after washing and solubilization with 2% NP-40.

Binding parameters were determined on bone marrow-derived macrophages under the conditions outlined above. Increasing concentrations of ^{125}I-MuIFN-gamma (Genentech) were incubated with macrophage monolayers in the absence or presence of a 100-fold excess of unlabelled IFN-gamma. Figure 2-4 represents an equilibrium saturation binding isotherm for macrophages. Specific binding was saturable at ligand concentrations above 1 nM (75 U/ml for this preparation). Scatchard analysis of the data indicated that there was a single class of approximately 11,500 binding sites per cell, with a dissociation constant (Kd) of 3.8×10^{-10} M (28 U/ml for this preparation).

Binding of ^{125}I-MuIFN-gamma to modulated macrophage populations. The ability to quantify receptor expression on macrophages that differ in their responsiveness to IFN-gamma provided the opportunity to determine whether these differences were mediated at the level of the receptor. Two models of macrophage responsiveness were examined. In the first, bone marrow-derived macrophages were pre-treated with a Type I interferon (in these experiments, MuIFN-β), which has been shown to significantly reduce the capacity of these macrophages to respond to IFN-gamma and bacterial lipopolysaccharide (LPS) for activation for tumor cell killing (129). Macrophages that had been exposed to IFN-β under conditions in which their response to IFN-gamma and LPS was substantially diminished

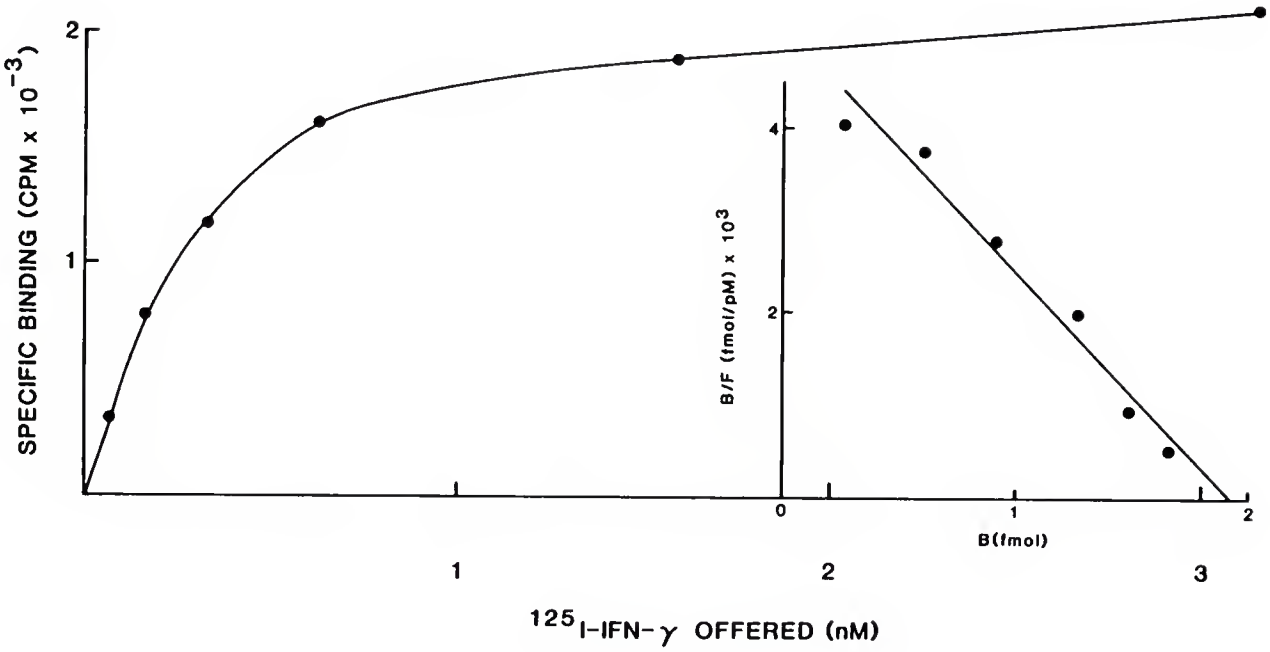

Figure 2-4. Equilibrium saturation binding of ^{125}I-MuIFN-gamma to macrophages.

Binding was performed as in Figure 2-2 for 2 hr at 37°C. The amount of ^{125}I-MuIFN-gamma specifically bound was plotted against the ratio of bound to free ligand to determine the affinity and number of binding sites (inset).

were examined for their ability to bind ^{125}I-MuIFN-gamma. MuIFN-β-treated macrophages bound slightly less ^{125}I-MuIFN-gamma (92%) than control cells, but the difference was minimal (Figure 2-5). No significant difference in affinity of binding was observed.

The second model of variation in macrophage responsiveness to IFN-gamma and LPS derives from studies of peritoneal exudate-derived macrophages. Macrophages derived from the peritoneal cavities of mice that have previously been injected with a sterile irritant (in this case, 1 ml fetal bovine serum containing 0.1 mg/ml LPS) are consistently more responsive to activating agents (IFN-gamma and LPS) than peritoneal macrophages from unmanipulated mice (Table 2-2). However, when these macrophages were compared for their IFN-gamma binding characteristics, no differences were observed either in the number of binding sites or in the affinity of binding (Table 2-2).

Binding of ^{125}I-MuIFN-gamma to primed and activated macrophages. To determine if the state of activation of macrophages affects IFN-gamma binding, bone marrow-derived macrophages were treated with IFN-gamma (primed) or IFN-gamma and LPS (activated) and tested for IFN-gamma binding (Figure 2-6). Prior to performing the binding assay, the cells were incubated with three successive 1.5-hour washes to allow any bound IFN-gamma to be internalized or

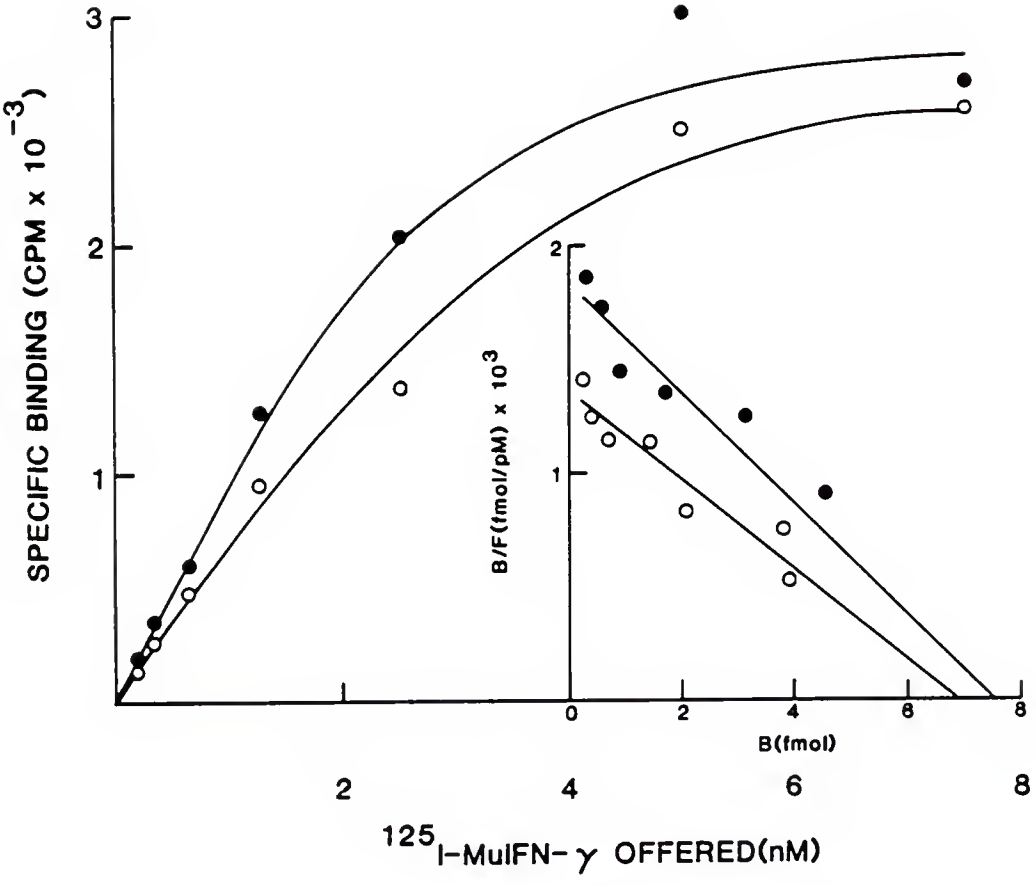

Figure 2-5. Effect of IFN-β pretreatment on specific binding of ^{125}I-MuIFN-gamma.

Bone marrow culture-derived macrophages were treated for 24 hr at 37°C with 100 U/ml IFN-β (o) or medium (●). Monolayers were washed and specific binding of ^{125}I-MuIFN-gamma (Schering) determined as in Figure 2-4. Binding parameters were determined by Scatchard analysis (inset). Control macrophages bound 7.55 femtomoles ^{125}I-MuIFN-gamma with a Kd of 4.15 nM, while IFN-β-treated cells bound 6.96 femtomoles with a Kd of 5.14 nM.

Table 2-2. Interferon Receptor Expression on Resident vs. Inflammatory Peritoneal Macrophages

	ED_{50} (U/ml)[a]			
	IFN alone	IFN + LPS	Receptors/ Cell[b]	Kd
Resident	42.0	1.02	35669 ± 3044	4.36×10^{-9}M
Inflammatory	6.1	0.16	34916 ± 2189	3.15×10^{-9}M

[a] ED_{50} refers to the concentration of gamma interferon, in the absence or presence of 3 ng/ml LPS, that induces 50% of the maximal tumoricidal response. Peritoneal exudate cells were harvested by lavage from C57BL/6 mice that were injected 24 hours earlier with 1 ml fetal bovine serum containing 0.1 ng/ml LPS (inflammatory) or were unmanipulated (resident). Cells were allowed to adhere to 96-well tissue culture plates (200,000 macrophages/well) and washed to remove nonadherent cells. stimuli were added for 6 hours, after which the monolayers were washed and exposed to ^{51}Cr-labelled P815 target cells for 16 hours.

[b] Specific binding of ^{125}I-MuIFN-gamma (Schering) was determined on monolayers in parallel with those used for cytotoxicity determinations.

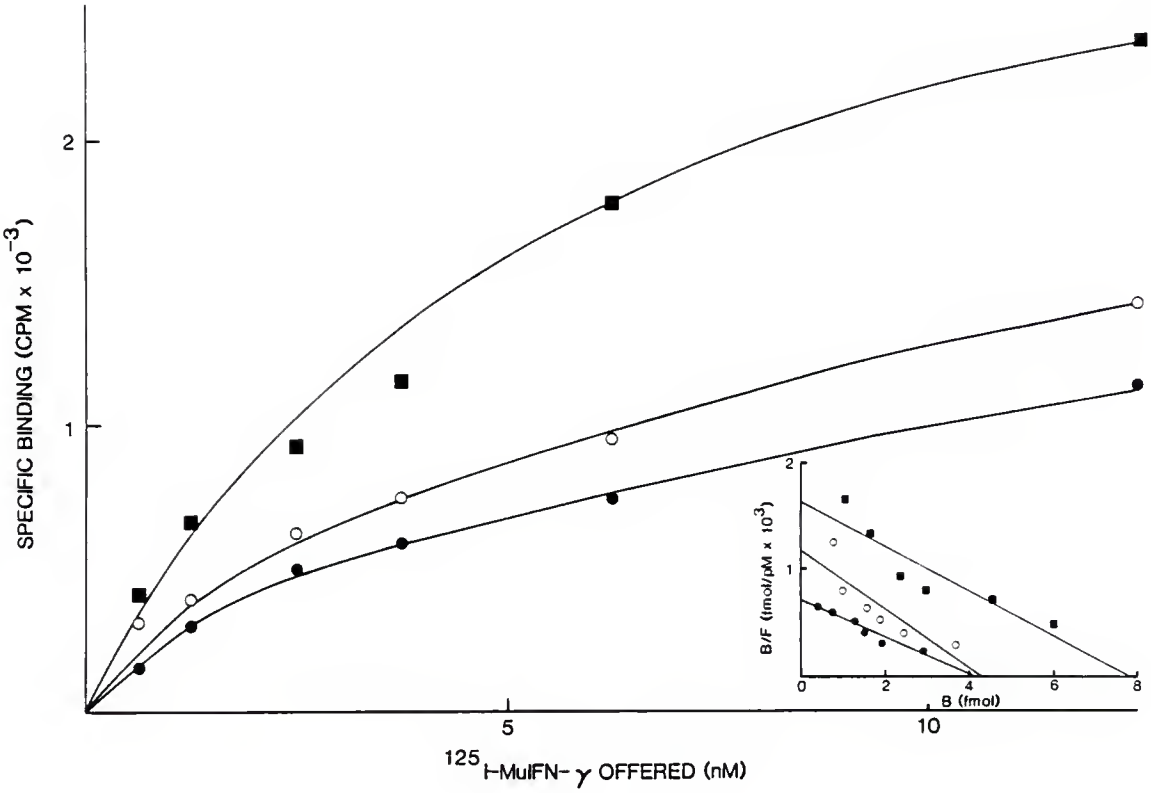

Figure 2-6. Binding of ^{125}I-MuIFN-gamma to primed and activated macrophages.

Bone marrow culture-derived macrophages were treated for 24 hr with either medium alone (■), 20 U/ml MuIFN-gamma (o), or 20 U/ml MuIFN-gamma + 1 ng/ml LPS (●). After three successive 1.5-hr incubations (37°C) in HMEM/10% FBS to remove and allow internalization of the MuIFN-gamma, macrophages were fixed with 0.1% paraformaldehyde and incubated with ^{125}I-MuIFN-gamma (Schering) to determine specific binding parameters. Femtomoles bound and dissociation constants were calculated: control, 7.80, 4.75 nM; primed, 4.17, 3.53 nM; activated, 4.01, 5.73 nM.

dissociated. Under these conditions, both primed and activated macrophages bound 50% less IFN-gamma than untreated controls. The reduction in binding was in receptor number, and no change in the affinity of binding was observed.

Discussion

The studies described here confirm the existence of specific, high affinity binding sites for mouse gamma-interferon on macrophages, and document the expression of these sites on different macrophage populations. These binding sites are believed to be receptors for IFN-gamma that represent the first step in the ultimate functional modulations of macrophages that are mediated by this ligand. While the complex nature of the pleiotropic responses of macrophages to IFN-gamma is not yet understood, the characterization of a specific receptor for this ligand is the necessary first step toward delineating the signals required for these varied responses.

Purified mouse gamma interferon, derived from *Escherichia coli* into which an expression vector containing the cDNA (54) had been introduced, was labelled with ^{125}I for use as a radioligand in binding experiments. Use of this radioligand as a priming stimulus in the induction of macrophage tumoricidal activity indicated that greater than 50% of the biological activity was retained. The specific

activity of the labelled IFN-gamma (≈10 µCi/µg) was quite high and similar to that reported by others (4,27,200).

The only two extant studies of IFN-gamma interaction with mouse macrophages have reported contrasting results (4,27). The data presented here support the work of Celada, et al. (27), i.e., that mouse macrophages, derived from bone marrow culture, possess a single class of relatively high affinity (0.4-4 nM, depending upon the MuIFN-gamma used) binding sites for IFN-gamma. Celada, et al. (27) reported a single class of IFN-gamma binding sites with a Kd of 1×10^{-8} M. In a later, more extensive report, however, Aiyer et al. proposed that macrophages possessed two classes of IFN-gamma receptors, one of intermediate affinity (3×10^{-9} M), and one of high affinity (9×10^{-11} M) (4). The latter was claimed to be the functional receptor, as its affinity was more consistent with physiologically active concentrations of the ligand. The results of the latter report were derived under conditions where internalization of the ligand was likely to have occurred; this is a more likely explanation of the curvilinear Scatchard plot derived in these studies, particularly in light of the extraordinary endocytic activity of macrophages (179). A recent report by Celada and Schreiber (29) documents the internalization of IFN-gamma by mouse macrophages; the data in Table 2-1 supports this finding.

The binding parameters derived in this study varied according to the preparation of MuIFN-gamma used. The Genentech preparation consistently yielded higher affinity estimates, with lower nonspecific binding. The Kd derived from the Schering preparations was roughly an order of magnitude lower (mid-nanomolar range) than the Genentech (Figures 2-5, 2-6, and Table 2-2). Differences of this magnitude are not unusual among IFN-gamma preparations (93). The specific antiviral activities of these preparations were reported by the suppliers as 2×10^7 and 1×10^6 U/mg for Genentech and Schering, respectively. In our hands, these activities were 5×10^6 and 2×10^6 U/mg, respectively. Differences in biological assay systems may have been responsible for these disparities.

For functional comparison, the ^{125}I-MuIFN-gamma used to derive the binding parameters in Figure 2-4 was directly assayed for biological activity (macrophage priming). The results indicate that priming can occur at concentrations well below half-maximal occupation of receptors (Kd = 28 U/ml). However, the concentrations of MuIFN-gamma required for priming are interdependent with the amount of triggering agent supplied as well (130). In addition, in strains of mice where MuIFN-gamma activates alone (or in the presence of undetectable amounts of a triggering agent), the concentrations required are low in comparison to the receptor affinity, suggesting that only a small

number of receptors need to be occupied for a significant functional response. The hypothesis has been advanced that multiple cycles of internalization of receptor-ligand complexes are required for the induction of the tumoricidal response (29). This would explain the fact that few receptors need to be occupied, as measured under static conditions, to elicit a complex response.

Studies of IFN-gamma receptor expression on different macrophage populations indicated that modulation of receptors is not likely the basis for differential responsiveness to IFN-gamma for tumoricidal activation. While some down-regulation of receptors on macrophages was apparent after pretreatment with Type I interferons, this change seemed insufficient to explain the drastic loss of responsiveness of the treated cells. The increased responsiveness of macrophages from inflammatory sites also appeared to be mediated at a level other than the IFN-gamma receptor. Resident and inflammatory macrophages were comparable on the basis of receptor number and affinity for IFN-gamma. These two models indicate that regulation of macrophage responses to IFN-gamma and triggering stimuli for tumoricidal activation is either mediated at a post-receptor level or at the level of the triggering stimulus (in this case, LPS). To date, there has been no evidence for regulation of IFN-gamma receptor expression by any stimuli that are known to affect macrophage functions.

The homologous ligand, IFN-gamma, does appear to affect the expression of its receptor. Macrophages that were either primed (treated with IFN-gamma alone) or activated (treated with IFN-gamma and LPS) both exhibited decreased binding of MuIFN-gamma. Binding of the ligand to these populations was reduced by about 50%. This "down-regulation" was specifically induced by IFN-gamma, and was independent of the state of activation. The decreased binding is not likely to be due to occupation of receptors by the MuIFN-gamma used to stimulate the cells, since 1) a very low concentration of MuIFN-gamma (20 U/ml) was used for priming and 2) the cells were washed with three successive 1.5-hour washes prior to the binding assay. These results are at odds with a report by Celada and Schreiber (29) that IFN-gamma-treated macrophages are not affected in their receptor expression. However, no data was shown in this report; direct comparison with these data were therefore impossible. The 4.5-hour incubation here, after removal of the ligand, probably allows internalization of occupied receptors that are not recycled in the absence of the ligand. Similar homologous ligand-induced receptor down-regulation has been reported for human IFN-α (22), platelet-derived growth factor (65), and epidermal growth factor (3), and is relatively common phenomenon (142).

These studies corroborate the presence of a specific, high affinity, single class of receptors for gamma interferon on mouse macrophages. They refute the previous claim of two distinct binding sites for MuIFN-gamma on macrophages (4), and indicate binding sites with an affinity at least 10-fold higher than that reported by Celada et al. (27). The examination of macrophage populations with altered responsiveness to activating stimuli indicated that IFN-gamma receptor expression is most likely not a major point of regulation for differences in capacity for activation. Finally, MuIFN-gamma appears to down-regulate its own receptor, although the functional consequences of such down-regulation are not readily apparent.

CHAPTER III

INTERACTIONS OF PRIMING AND TRIGGERING STIMULI WITH MACROPHAGES AS REVEALED BY CELL SURFACE PROTEOLYSIS

Introduction

Macrophages can be activated to kill neoplastic cells as the result of a series of steps induced by a sequence of stimuli (68,108,150). Quiescent macrophages first reach an inflammatory stage (1,68,108), then become primed to respond to a final triggering signal to become fully cytolytic. While stimuli inducing the inflammatory stage are largely undefined, priming and triggering agents have been well-documented. The only known priming agents are the interferons. Of these, gamma interferon (IFN-gamma) is much more potent for priming than α/β interferons when they are compared on the basis of antiviral units (131). Triggering stimuli can be provided by a number of substances, including bacterial lipopolysaccharide (LPS) (5,130), heat-killed Listeria monocytogenes (HKLM) (165), and polyinosinic-polycytidylic acid (poly I:C) (183,185). When used at high concentrations, these agents can activate macrophages fully; however, direct activation of this nature still occurs by a two-step process, in that the

triggering agents induce α/β interferon production by the macrophage, which then primes in an autocrine manner (166). While the phenomenon of macrophage activation is well-characterized (1), little is known about its induction at the subcellular level.

The first step in the priming of macrophages by murine IFN-gamma (MuIFN-gamma) most likely requires the engagement of a specific receptor for this lymphokine (27,151,163). Little is known, however, about how the various triggering agents provide their signal for the induction of cytolytic activity. The results presented here will demonstrate that binding of ^{125}I-MuIFN-gamma and the induction of activation are affected differently by prior treatment of macrophages with various proteolytic enzymes. The data indicate that, in addition to engagement of the MuIFN-gamma receptor, other cell surface structures are required for activation. Proteolysis also differentiated between two means of triggering primed macrophages, and dissociated two induced proteins, previously thought to be specific for the triggering response, from the triggering phenomenon.

Materials and Methods

Media and reagents. All experiments were performed in HEPES-buffered, modified Eagle's medium (HMEM). HMEM consisted of modified Eagle's minimum essential medium (Auto-Pow MEM) prepared with 2 mg/ml sodium bicarbonate, 2

mM glutamine (all from Flow Laboratories, McLean, VA), 100 U/ml injectable penicillin G potassium, 100 µg/ml injectable streptomycin (both from Pfizer, Inc., New York, NY), and 15 mM N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES; Sigma Chemical Co., St. Louis, MO). For experiments, HMEM was supplemented with 10% v/v fetal bovine serum (FBS; Sterile Systems, Inc., Logan, UT). Heparin sodium (O'Neal, Jones, and Feldman, St. Louis, MO) was used at 2 U/ml in HMEM for bone marrow harvest. For bone marrow culture, HMEM was supplemented with 15% v/v L cell-conditioned medium, 10% v/v FBS, and 5% v/v horse serum (Flow Laboratories, McLean, VA). L cell conditioned medium was obtained from cultures of NCTC L929 cells grown at confluency for 4 days in HMEM/10% FBS, and filtered (0.45 µm) before use. All media tested negative for the presence of endotoxin by the Limulus amebocyte lysate (LAL) (Associates of Cape Cod, Inc., Woods Hole, MA) assay (97) at a sensitivity of 0.07 ng/ml. Purified, lipid A-rich fraction II of phenol-extracted bacterial lipopolysaccharide (LPS) from Escherichia coli 0111:B4 (116) was a gift from Dr. D. C. Morrison, Department of Microbiology, University of Kansas School of Medicine, Kansas City, KS. Heat-killed Listeria monocytogenes was a gift from Dr. R. D. Schreiber, Washington University, St. Louis, MO. Polyinosinic-polycytidylic acid was from Calbiochem (San Diego, CA).

Bone marrow culture. Macrophages were obtained from bone marrow cultured as described by Leung et al. (96) as modified from Meerpohl et al. (107). Bone marrow was harvested from the tibiae and femora of 6-9 week old C3H/HeN mice (Charles River Laboratories, Kingston, NY) and cultured in bacterial grade 100 mm^2 Petri dishes (Lab-Tek Products, Naperville, IL) at 37° C, in an atmosphere containing 5% CO_2. Bone marrow was seeded at 4 x 10^6 nucleated cells/dish in 15 ml of bone marrow culture medium. On day 7, 5 ml of the same medium were added to each dish. Cells were harvested for use between days 11 to 13 of culture using ice cold phosphate-buffered saline (PBS). Differential analysis of cytocentrifuge preparations stained with Diff-Quik (Harleco, Gibbstown, NJ) showed that populations of cells derived in this manner consistently contained 97-99% macrophages.

Interferon. Recombinant mouse gamma interferon (MuIFN-gamma) was produced by E. coli into which an expression vector had been introduced that contained the MuIFN-gamma coding sequence (54). This reagent was a gift either from Genentech, Inc. (lot #1551/43; South San Francisco, CA) or from Schering-Plough Corp. (lots #18501-131 PF 28-33 and 6-M6-01; Bloomfield, NJ).

Proteolytic enzymes. Pronase (protease Type XIV from Streptomyces griseus), trypsin (Type III, from bovine pancreas), and chymotrypsin (Type VII, TLCK-treated, from

bovine pancreas) were all obtained from Sigma. The specific caseinolytic activities determined for these enzymes were 3.4 U/mg, 5.7 U/mg, and 6.4 U/mg, respectively, where a unit was defined as the ability to produce 1 µmole of tyrosine equivalents from the substrate (0.65% casein, w/v) per minute at 37°C.

Radioiodination of MuIFN-gamma. MuIFN-gamma was radioiodinated using either immobilized glucose oxidase-lactoperoxidase (Enzymobeads; BioRad, Richmond, CA), according to the manufacturer's instructions, or the chloramine T method (71). The labeled preparations were stored at 4°C after addition of 2-mercaptoethanol to 0.1% (v/v). Each radioiodinated preparation was tested for biological activity by measuring its ability to prime macrophages for tumor cell killing (see below). The results were used to calculate the specific activity of each preparation using unlabelled MuIFN-gamma as a reference. Specific activities ranged from 10-50 µCi/µg with greater than 50% recovery of biological activity.

Assay for macrophage priming and activation. Priming was defined as acquisition by macrophages of the capacity to respond to a second, triggering signal (in this system, either LPS, heat killed Listeria monocytogenes [HKLM], or poly I:C) by developing cytolytic activity for tumor cells. To quantify priming activity (130), monolayers of bone marrow culture-derived macrophages (5 x 10^4/well in 96-well

microtiter plates) were incubated at 37°C with different concentrations of MuIFN-gamma in the absence or presence of the triggering stimulus, for the times indicated in the text. After the incubation period, monolayers were washed with HMEM/10% FBS. ^{51}Cr-labelled ($Na_2{}^{51}CrO_4$, 300 mCi/mg; Amersham Corp., Arlington Heights, IL) P815 mastocytoma cells were then added in 200 µl HMEM/10% FBS as targets (2 x 10^4/well). After 16 hr at 37°C, the upper 100 µl of supernate were removed and analyzed in an automatic gamma spectrometer to determine the extent of ^{51}Cr release. Cytotoxicity was expressed as % specific ^{51}Cr release as calculated using the formula:

$$\frac{\text{experimental cpm-spontaneous release cpm}}{\text{total releasable cpm-spontaneous release cpm}} \times 100$$

Total releasable cpm were determined after freeze-thawing under hypotonic (half distilled water) conditions. Spontaneous release was that which occurred when target cells were held throughout the incubation period on unstimulated monolayers of macrophages.

Assay for binding of ^{125}I-MuIFN-gamma. Macrophage monolayers were prepared exactly as described above. After allowing cells to adhere, they were washed and exposed to 50 µl of 100 mM NaN_3 in HMEM/10% FBS for 15 minutes. In pilot studies, macrophages that were exposed to 100 mM NaN_3 for 15 minutes and then maintained in 50 mM NaN_3 stopped

all detectable nonspecific endocytosis (using horseradish peroxidase as a marker) and recovered endocytic activity in NaN_3-free medium. An equal volume containing ^{125}I-MuIFN-gamma was next added to wells in the presence or absence of a 100-fold excess of unlabelled MuIFN-gamma. In pilot experiments, this concentration of unlabelled MuIFN-gamma inhibited binding by >90%. Incubation at 37°C for 2 hours was followed by 3 rapid washes in ice-cold HMEM/10% FBS. Cells were then solubilized in 1% SDS, harvested, and lysates were analyzed in an automatic gamma spectrometer.

Detection of proteins induced by activating agents. The induction of specific newly synthesized proteins in macrophages treated with various stimuli was performed as described by MacKay and Russell (103). Macrophage monolayers were simultaneously exposed to stimuli (IFN-gamma and triggering agents) and 20 µCi L-[^{35}S] methionine (Specific activity = 1060 Ci/mmol; Amersham, Arlington Heights, IL) for 6 hr in methionine/leucine/lysine-free HMEM + 10% fetal bovine serum. After labelling, monolayers were washed three times in phosphate-buffered saline and solubilized in a lysis solution composed of 9.5 M urea, 2% (w/v) NP-40, 100 mM dithiothreitol, 2% w/v ampholytes, pH 3.5-10 (Ampholines, LKB Instruments, Gaithersburg, MD) and protease inhibitors phenylmethylsulfonylfluoride and pepstatin A. Lysates were immediately frozen and stored at -70°C. Trichloroacetic acid-precipitable radioactivity was

determined, and equal counts (usually 1.5 x 10^6 CPM) were analyzed by two-dimensional gel electrophoresis as modified from O'Farrell (126). Samples were subjected to isoelectric focusing in the first dimension, in 2.5 x 125 mm tube gels containing 4.24% w/v acrylamide, 0.74% DATD, 9.5 M urea, 2% w/v NP-40, and 2.88% w/v ampholytes (2.4% pH 5-7 and 0.48% pH 3.5-10). After focusing for 7000 volt-hours, first-dimension gels were equilibrated in SDS sample buffer and frozen (-70° C) until used for the second dimension, SDS-polyacrylamide gel electrophoresis. Tube gels were subjected to SDS-PAGE on 160 mm wide x 280 mm long x 1.6 mm thick resolving slab gels (10% w/v acrylamide with 0.27% w/v bis-acrylamide). Electrophoresis was performed at 11 mA constant current, then processed for fluorography after fixation in TCA. Processing was performed by immersing gels in two successive baths of dimethylsulfoxide to dehydrate them, followed by impregnation with 2,5-diphenyloxazole for 1.5 hours. After rehydration of gels to remove DMSO, gels were dried and exposed to XAR-5 radiographic film (Kodak, Rochester, NY) for 1.5 x 10^6 CPM-days.

Results

Time course of macrophage activation for tumor cell killing. Bone marrow-derived macrophages were exposed for various times to various concentrations of MuIFN-gamma with

or without a constant amount of LPS (1 ng/ml) (Figure 3-1). As expected, with either MuIFN-gamma or LPS used alone at such a low concentration, no killing was observed. A response of the macrophages to MuIFN-gamma in the presence of 1 ng/ml LPS was observed. It was both dose and time-dependent. At concentrations of 5 U/ml and above, stimulation was maximal and the responses, therefore, indistinguishable. In each instance they began to develop after 2 hr and peaked by 6 hr. In contrast, at the lower concentration of 1 U/ml, the response developed at the same rate, but only after an initial lag period of 2 additional hr. As expected (108), the tumoricidal capacity of all monolayers gradually decreased with continuing exposure to the activating agents. All subsequent experiments were performed with a 6-hr stimulus exposure to achieve the maximal response at appropriate concentrations of stimuli.

Effect of proteases on macrophage activation for tumor cell killing. Three proteolytic enzymes with different specificities (trypsin cleaves after arginines and lysines, chymotrypsin after aromatic amino acids, and Pronase is broadly active, cleaving bonds between many different amino acids) were investigated to determine whether or not they would affect responsiveness of macrophages to MuIFN-gamma and LPS. Macrophage monolayers were pre-treated with either Pronase, trypsin, or chymotrypsin in serum-free medium for the periods of time indicated on the abscissa.

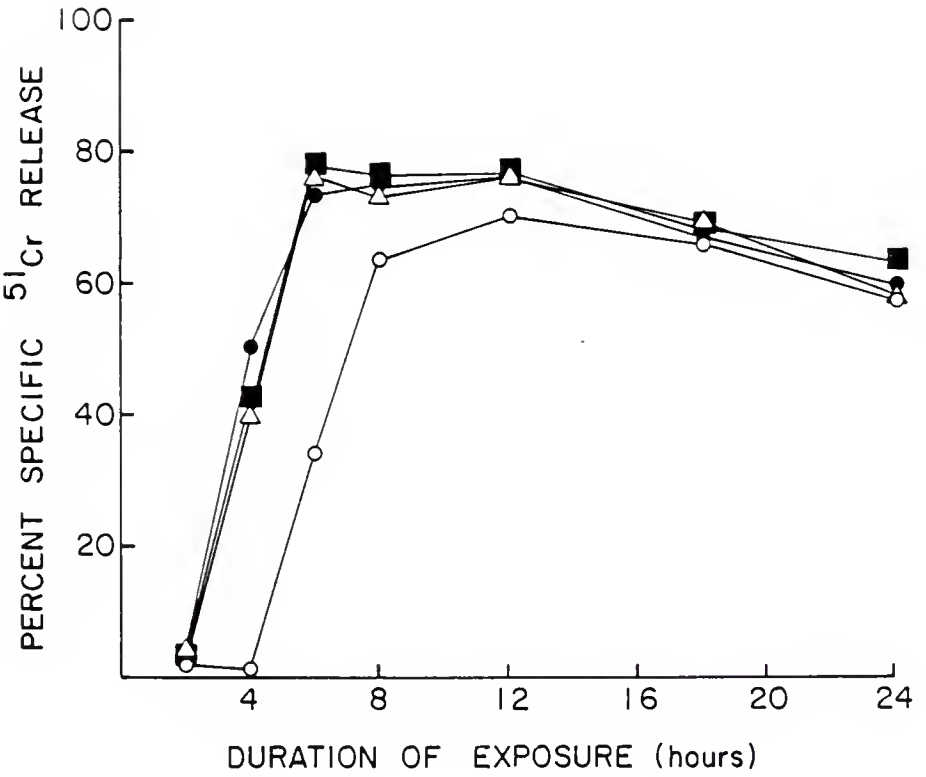

Figure 3-1. Time course of macrophage activation by MuIFN-gamma in the presence of LPS.

Bone marrow culture-derived macrophages were seeded in 96-well tissue culture plates and allowed to adhere for 2 hr at 37°C. Monolayers were washed and MuIFN-gamma was added at 1 (o), 5 (Δ), 10 (■), or 50 (●) U/ml in the presence or absence of 1 ng/ml LPS for the times indicated on the abscissa. Stimuli were removed, the monolayers were washed, and ^{51}Cr-labelled P815 target cells were added for an additional 16 hr to determine cytolytic activity by ^{51}Cr release. No killing was observed in the absence of LPS or with LPS alone.

The monolayers were then washed and incubated for 5 min in 20% FBS to minimize residual proteolytic activity. ^{125}I-MuIFN-gamma was then added, with or without 1 ng/ml LPS. Exposure to each of the proteases resulted in a reduction in the capacity of macrophages to become activated to a tumoricidal state (Figure 3-2). However, the enzymes had different effects. All three had a marked, initial effect, although Pronase caused the most pronounced reduction in killing. After 30 min the magnitude of the cytolytic response to activators continued to be reduced, but at a different rate than that which had been observed earlier.

Effect of proteases on specific binding of ^{125}I-MuIFN-gamma. To determine if the reduction of macrophage responsiveness to activating stimuli was due to decreased binding of MuIFN-gamma, binding experiments were performed in parallel with the functional studies. The same pools of reagents and cells were used to optimize comparison between the two kinds of experiments. Figure 3-3 shows the effects of the three proteases on the capacity of macrophages to bind ^{125}-I-MuIFN-gamma. While trypsin and chymotrypsin had had a significant effect on responsiveness (Figure 3-2), these enzymes had no detectable effect on the binding of ^{125}I-MuIFN-gamma. Pronase-treated macrophages, on the other hand, bound approximately 40% less interferon, and most of the loss occurred within the first 30 min of incubation. Equilibrium saturation binding analysis over a

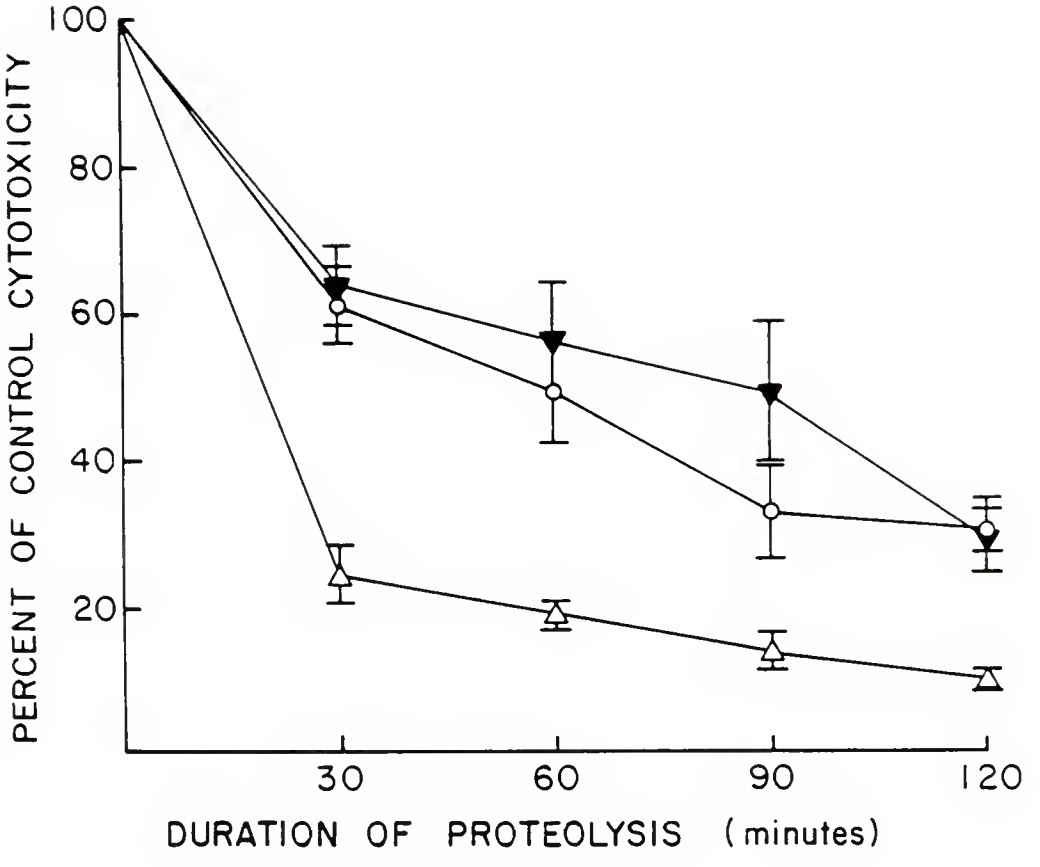

Figure 3-2. Effect of proteases on responsiveness of macrophages to MuIFN-gamma and LPS.

Bone marrow culture-derived macrophage monolayers were prepared in serum-free medium and exposed to 50 µg/ml Pronase (Δ), trypsin (o), or chymotrypsin (▼) at 37° C for the indicated times. Based on the caseinolytic activities of these enzymes, this represented 0.170, 0.285, and 0.320 U/ml, respectively. After protease treatment, cells were washed and incubated in medium containing 20% fetal bovine serum (FBS) for 5 min at 37° C to minimize residual proteolytic activity. The cells were then exposed to 50 U/ml MuIFN-gamma + 1 ng/ml LPS for 6 hr, washed to remove the stimuli, and incubated for an additional 16 hr with ^{51}Cr-labelled P815 target cells to determine cytolytic activity. Results are means ± 1 SEM from two separate experiments (n = 4).

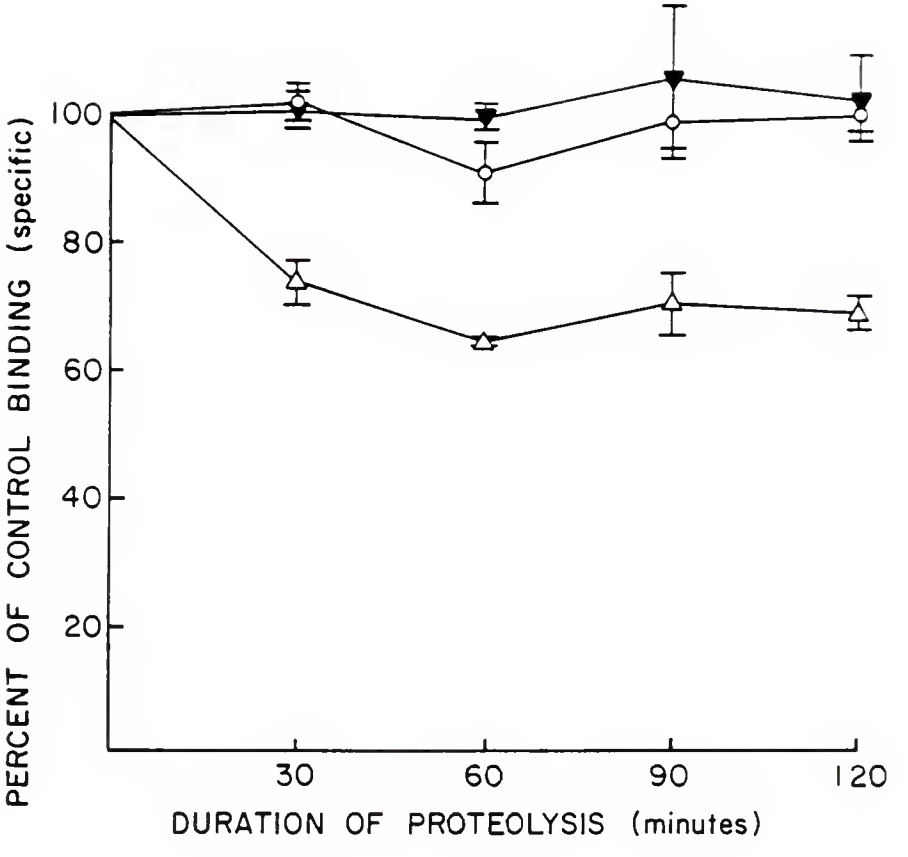

Figure 3-3. Effect of proteases on specific binding of ^{125}I-MuIFN-gamma.

Macrophage monolayers were treated in parallel with those from Figure 3-2 with 50 µg/ml Pronase (Δ), trypsin (o), or chymotrypsin (▼). Specific binding of ^{125}I-MuIFN-gamma was then determined as described in Materials and Methods, at a concentration of 50 U/ml ^{125}MuIFN-gamma ± 5000 U/ml unlabelled MuIFN-gamma.

range of ^{125}I-MuIFN-gamma concentrations indicated no decrease in either the affinity of binding or the number of binding sites as a result of trypsin treatment, even when the concentration of trypsin was increased 10-fold (to 500 µg/ml) (data not shown).

Regeneration of responsiveness to activating stimuli by Pronase-treated macrophages. To ensure that the effects on responsiveness were not due to a trivial explanation, e.g., a cytotoxic effect on the macrophages, experiments were undertaken to determine if protease-treated monolayers could regenerate their capacity to respond to activating stimuli. Pronase was chosen for these experiments because it had previously had the most extensive effect. As can be seen in Figure 3-4, control macrophages began to express cytolytic activity in response to MuIFN-gamma and LPS stimulation after 4 hr, while monolayers pretreated with Pronase became responsive to MuIFN-gamma and LPS after an additional lag period of about 8 hr. At that time, treated macrophages began to develop the capacity to kill at the same rate and ultimately reached the same maximal level of cytolytic activity that the untreated controls had.

Regeneration of binding of ^{125}I-MuIFN-gamma by protease-treated cells. Experiments were also performed to determine if the binding capacity of Pronase-treated macrophages could be regenerated. Macrophage monolayers were treated as before, but with 500 µg/ml Pronase, an

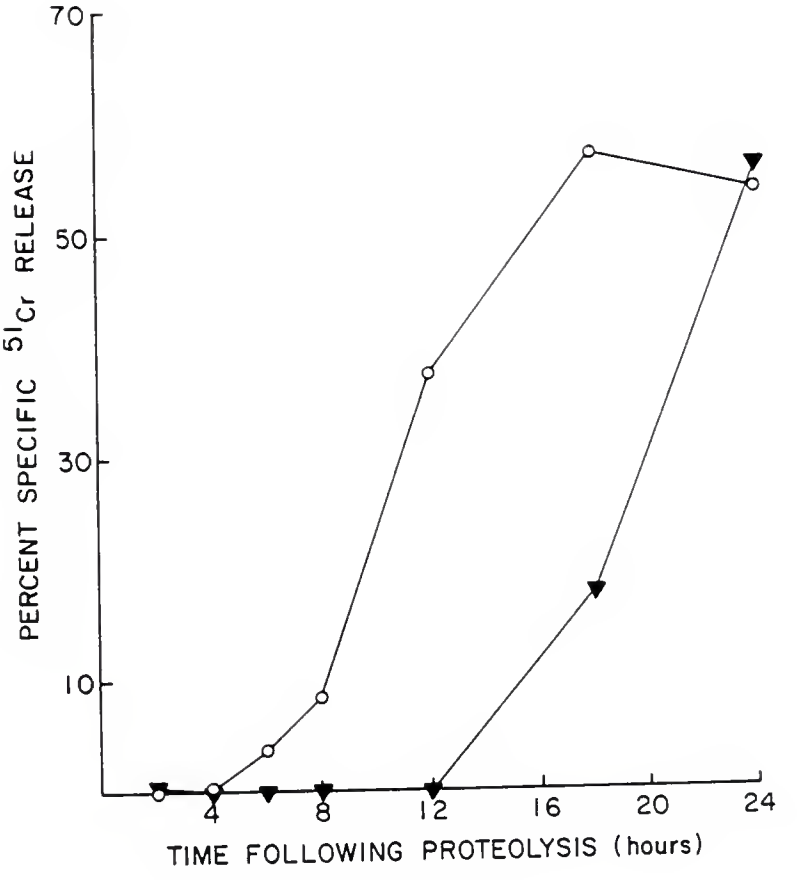

Figure 3-4. Regeneration of responsiveness of Pronase-treated macrophages to MuIFN-gamma and LPS.

Macrophage monolayers were treated with 500 µg/ml Pronase (▼) or medium alone (o) for 2 hr, after which they were washed and incubated in 20% FBS for 5 min. Macrophages were immediately exposed to 50 U/ml MuIFN-gamma ± 1 ng/ml LPS in HMEM/10% FBS for the times indicated on the abscissa. At those times, monolayers were washed to remove stimuli, and ^{51}Cr-labelled P815 target cells were added to determine cytolytic activity.

amount that was shown in pilot experiments to nearly eliminate binding of MuIFN-gamma (10% of specific binding on control cells; data not shown). After exposure to the protease, cells were incubated for varying times in either medium alone or medium that contained 20 µg/ml cycloheximide, and specific binding was quantified at each time point. As is shown in Figure 3-5, specific binding capacity for MuIFN-gamma began to return after approximately 2 hr and peaked by 4 hr. Recovery did not occur in the presence of cycloheximide although macrophages remained viable (trypan blue dye exclusion as the criterion).

Status of trypsin-treated macrophages. Further experiments were performed to determine the nature of the trypsin-induced decrease in responsiveness of macrophages. Macrophage monolayers were pretreated with 500 µg/ml (2.85 caseinolytic U/ml) trypsin for 1 hour, then exposed to a combination of MuIFN-gamma and three different triggering agents (LPS, HKLM, and poly I:C) to determine if the loss of responsiveness was a generalized phenomenon or due to a selective loss of responsiveness to triggering stimuli. Dose-response cytotoxicity data are shown in Figure 3-6 for each of the triggering agents in the presence of a constant amount of MuIFN-gamma (10 U/ml). In each case, macrophages responded to the triggering agents in a dose-dependent manner. No killing was observed with the triggers alone

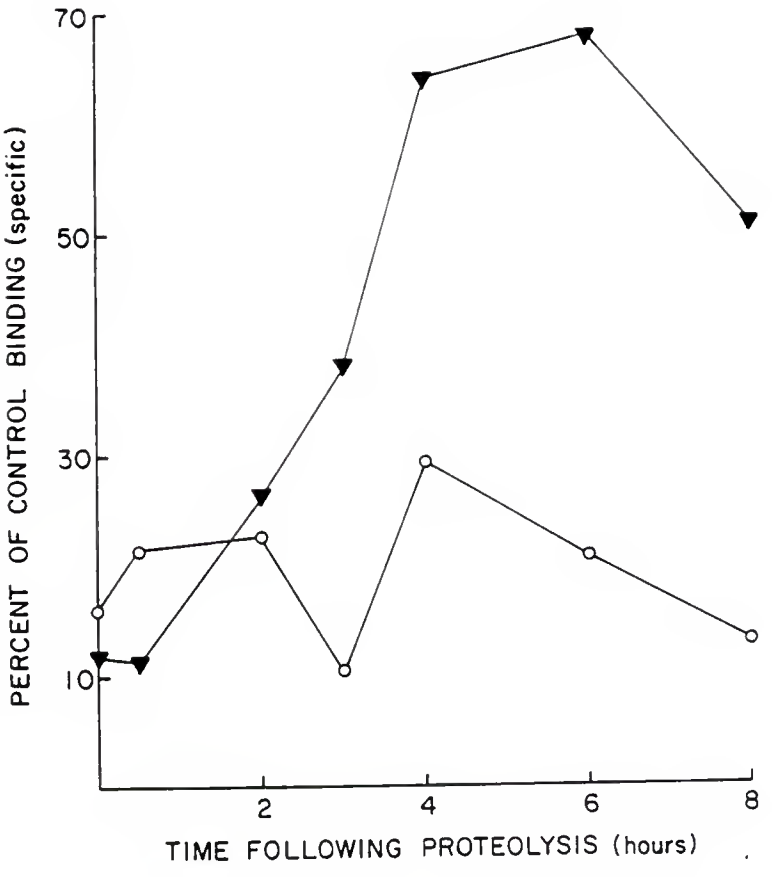

Figure 3-5. Regeneration of binding of ^{125}I-MuIFN-gamma by Pronase-treated macrophages.

Macrophage monolayers were treated with 500 µg/ml Pronase in serum-free medium for 2 hr at 37°C. After washing and incubation in 20% FBS for 5 min, cells were allowed to recover in the absence (▼) or presence (o) of 20 µg/ml cycloheximide in HMEM/10% FBS for the times indicated on the abscissa. Monolayers were then washed and specific binding of ^{125}I-MuIFN-gamma determined.

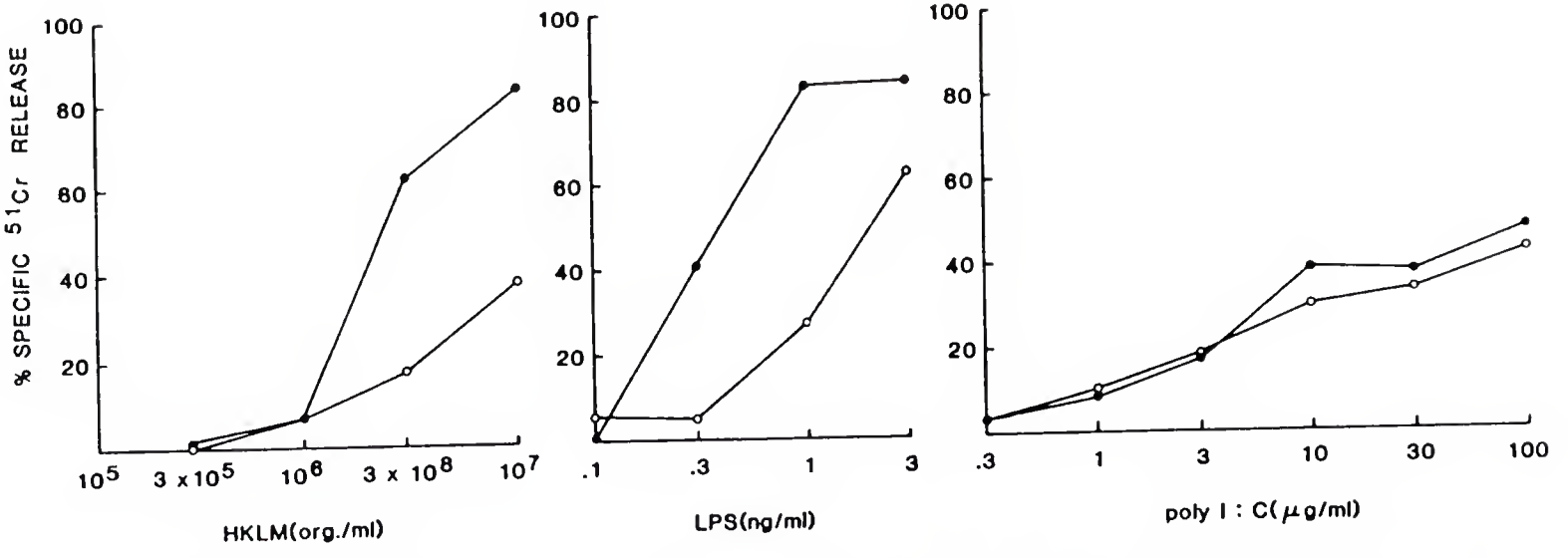

Figure 3-6. Effects of trypsin pre-treatment on macrophage activation by MuIFN-gamma in the presence of LPS, HKLM, or poly I:C.

Macrophage monolayers were treated with 500 µg/ml (2.85 U/ml) trypsin (o) or serum-free medium alone (●) for 1 hr at 37° C. After washing and incubation for 5 min with 20% FBS, cells were exposed to MuIFN-gamma (10 U/ml) in the presence of the indicated concentrations of LPS, HKLM (in organisms/ml), or poly I:C for 6 hr at 37° C. Monolayers were then washed to remove stimuli, and ^{51}Cr-labelled P815 target cells added for an additional 16 hr to determine cytotoxicity. No killing was observed in the presence of MuIFN-gamma or any of the triggering agents alone.

under these conditions, or with MuIFN-gamma alone. The slope of the dose-response curve for poly I:C was much more shallow than that for either LPS or HKLM, which yielded similar response curves. If sufficient triggering stimulus was added, the trypsin-treated macrophages were fully cytolytic compared to controls, indicating that the effect was one of reduction of responsiveness to LPS or HKLM, rather than a general cytotoxic effect on the macrophages or total loss of responsiveness. The most striking observation, however, was that, while the triggering responses to LPS and HKLM were substantially abrogated by prior trypsin treatment, the response to poly I:C was unaffected.

To further ascertain the response of trypsin-treated macrophages to activating agents, treated and control cells were exposed to stimuli in the presence of ^{35}S-methionine and examined for induced proteins as described by MacKay and Russell (103). It was determined in pilot experiments that the trypsin preparations used in these experiments could trigger macrophages in the presence of MuIFN-gamma, and that this activity was inhibitable by polymyxin B (data not shown); this indicated that the trypsin was contaminated with endotoxin. To avoid the complications of endotoxin-induced proteins by this contaminating endotoxin, C3H/HeJ (endotoxin-hyporesponsive) macrophages were used in these experiments. These mice are responsive to HKLM as a triggering agent. Medium- and trypsin-treated macrophages

were exposed to 10 U/ml MuIFN-gamma in the presence of various concentrations of HKLM in methionine-free HMEM/10% containing either ^{35}S-methionine (for lysate analysis) or an equal volume of PBS (for cytotoxicity determination). Lysates from populations of macrophages that expressed large differences in tumoricidal activity at the same doses of stimuli (70 and 17% for control and trypsin-treated macrophages, respectively) were analyzed by two-dimensional gel electrophoresis. Fluorograms of these gels are depicted in Figure 3-7. The expression of p47b, a protein previously shown to be associated with macrophage priming (103), was intact in both control (B) and trypsin-treated (C) macrophages. The expression of p71/73, a pair of coordinately regulated proteins previously shown to be induced by triggering stimuli, was also unaffected, despite large differences in tumoricidal activity. The expression of other proteins associated with induction by these stimuli was also unchanged in these macrophages. Neither p47b nor p71/73 was induced in C3H/HeJ macrophages pre-treated with trypsin and then incubated in medium alone (data not shown).

Discussion

Macrophage activation for tumor cell killing usually occurs in a stepwise fashion as the result of exposure to multiple stimuli. The two stages under investigation here,

Figure 3-7. Effects of trypsin pre-treatment on the induction of priming- and triggering-specific proteins.

C3H/HeJ bone marrow culture-derived macrophages were treated with 500 µg/ml trypsin for 1 hr at 37°C, washed, and incubated with 20% FBS for 5 min. MuIFN-gamma (10 U/ml) was then added in the presence of various concentrations of heat-killed Listeria monocytogenes (HKLM) for 6 hr, and cytotoxicity determined as before. At the concentration of 1 x 10^6 organisms/ml, cytotoxicity by control and trypsin-treated macrophages was 70% and 17%, respectively. Two-dimensional gel electrophoresis was used to analyze induced proteins from macrophages labelled with ^{35}S-methionine for 6 hr in parallel with those used for the cytotoxicity determination. Panel A is from macrophages incubated in medium alone; panels B and C are from the control and trypsin-treated macrophages, respectively, stimulated with 10 U/ml MuIFN-gamma + 10^6 HKLM/ml.

A
p71,73
p47b
B
p71,73
p47b
C
p71,73
p47b

priming and triggering, represent the final steps in the modulation of a complex function (tumoricidal activity). Understanding the subcellular events required for the full induction of activation is of primary importance in revealing the mechanisms behind the regulation of macrophage tumoricidal function. The results reported here confirm the requirement for a cell-surface receptor for gamma interferon for priming for cytolytic activity in macrophages, and implicate the requirement for other protease-labile structures in the triggering response as well. These results also suggest the existence of at least two different pathways for triggering of primed macrophages, and separate the induction of triggering agent-induced proteins from the functionally important triggering event.

The existence of specific cell-surface receptors for IFN-gamma on macrophages has been documented for both mouse (4,27,89) and human (26,50,128,143) cells. There have been few data, however, to directly demonstrate the functional requirement for these receptors for macrophage priming, with the exception of the effects of blocking antibodies (151,163). The data shown here demonstrate that the MuIFN-gamma receptor on macrophages is susceptible to proteolysis by a broad spectrum protease (Pronase), and that the digestion of the receptor is accompanied by a loss of macrophage responsiveness to the ligand. This confirms

that the receptor is, at least in part, a protein and the fact that it is essential for the induction by MuIFN-gamma of non-specific tumor cell killing by macrophages. The binding activity for ^{125}I-MuIFN-gamma returned fully after removal of the protease within 4 hours; this recovery was dependent upon de novo protein synthesis, as it did not occur in the presence of cycloheximide (Figure 3-5). This shows that 4 hours is the period of time required to synthesize and express MuIFN-gamma receptors on the cell surface. In addition, these data indicate that the receptor is constitutively synthesized. Pronase-treated macrophages did not recover their capacity to express cytolytic activity in response to MuIFN-gamma and LPS for another 4 hr, i.e., until 8 hours after removal of the enzyme (Figure 3-4). This is consistent with the fact that, once functional receptors are available, and at concentrations of stimuli that are fully stimulatory, it requires approximately 4 hours to generate the cytolytic response (Figure 3-1). In addition to the MuIFN-gamma receptor, any other protease-labile structures required for activation would have to be regenerated within this same time.

In contrast to the human IFN-gamma receptor (26), the MuIFN-gamma receptor does not appear to be susceptible to trypsin digestion. This fact was confirmed at three levels. First, specific binding of ^{125}I-MuIFN-gamma was

unaffected by trypsin treatment (Figure 3-3). Second, the response of trypsin-treated macrophages to MuIFN-gamma for induction of the interferon-induced protein, p47b remained intact (Figure 3-7). Finally, the functional response of these cells to MuIFN-gamma was also unaffected, as they readily became activated if provided the proper triggering agent (Figure 3-6; poly I:C panel).

A novel finding revealed by these experiments is the distinction of triggering responses between LPS/HKLM and poly I:C. Earlier studies by Taramelli and Varesio (183) suggested that such a difference exists; they found that resident peritoneal macrophages were refractory to LPS but not to poly I:C activation. The resistance of the poly I:C response to trypsin treatment (Figure 3-6) indicates that this triggering agent probably acts via a pathway different from that of LPS or HKLM. These data also suggest that LPS and HKLM act by a similar, if not identical, mechanism that is susceptible to alteration by trypsinization. Trypsin treatment affected the HKLM response equally in both C3H/HeN and C3H/HeJ macrophages. If LPS and HKLM interact with the same trypsin-sensitive structure, this would suggest that the LPS hyporesponsiveness in HeJ macrophages is due to some subtle alteration of this structure, perhaps resulting in a lower affinity for the LPS molecule. Presumably, the post-receptor (for HKLM) mechanisms are intact in these cells. This is an important finding with

respect to understanding the lack of LPS-responsiveness of C3H/HeJ macrophages. Finally, these results have separated the induction of expression of LPS/HKLM-induced proteins (specifically p71/73) from the actual triggering event (Figure 3-7). This finding is compatible with the fact that triggering of primed macrophages for induction of cytolytic activity is defined by a rapid (<15 minutes) response that would not be expected to depend upon the synthesis of new proteins. Trypsin affected the triggering response without affecting the LPS/HKLM-induced proteins p71/73, as well as others that have previously been linked to LPS stimulation of macrophages (103). There are at least two possible explanations for this result. 1) Trypsin is digesting a cell-surface protein(s) required for the triggering response, but not for the induction of p71/73 and other LPS-induced proteins. The shift in dose-responses to triggering stimuli rather than a complete loss of response suggests that trypsin may not be completely digesting these structures, but cleaving them so as to reduce their affinity for the ligands (LPS, HKLM). 2) Trypsin acts in a pharmacological manner, providing or inducing a signal that negatively regulates triggering by LPS or HKLM. The enhancement of C3H/HeJ B-cell responsiveness by trypsin treatment has led Kuus et al. (86) to speculate that LPS activates a membrane-associated protease that results in signal transduction for B-cell

proliferation. In either case, these data suggest the importance of a trypsin-labile substrate that is necessary for LPS responses, particularly triggering for tumoricidal activity.

In conclusion, the use of cell surface proteolysis has revealed several observations related to the induction of macrophage activation. 1) The receptor for MuIFN-gamma is resistant to functional alteration by trypsin, in contrast to the human receptor (26). 2) The MuIFN-gamma receptor, in addition to other trypsin-labile structures, is required for the induction of activation by MuIFN-gamma in the presence of LPS or HKLM. 3) Lipopolysaccaride or HKLM and poly I:C appear to act via two distinct pathways for triggering of primed macrophages. 4) The triggering response has been distinguished from the induction of proteins synthesized in the presence of these same stimuli.

CHAPTER IV

SOLUBILIZATION AND QUANTIFICATION OF THE RECEPTOR FOR MURINE GAMMA INTERFERON

Introduction

Gamma interferon, a product of stimulated T lymphocytes, induces multiple alterations in the function and characteristics of mononuclear phagocytes. These result in an overall enhancement of the host defense functions performed by these cells (reviewed in 1,161,192, 203). Among the more complex changes regulated by this lymphokine is the development of tumoricidal activity (133,164). Characterization of a functional receptor for gamma interferon on macrophages is the first step toward understanding how it induces activation for tumor cell killing. To isolate and definitively characterize the binding site, the receptor must first be solubilized in an active form, and a means found to quantify binding activity during subsequent purification steps. The work to be described here has successfully addressed these needs, using as starting material the mouse myelomonocytic cell line, WEHI-3. Gamma interferon binding activity was detected in detergent-solubilized WEHI-3 membrane lysates by a liposome-based precipitation assay. Use of this

soluble receptor assay confirmed the expectation that IFN-gamma binding activity could be significantly enriched by affinity chromatography on IFN-gamma-Sepharose.

Materials and Methods

Cell Culture and membrane preparation. WEHI-3 cells were passed daily in 1500-ml spinner flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mg/ml sodium bicarbonate, 2 mM glutamine (all from Flow Laboratories, McLean, VA), 100 U/ml penicillin G potassium, 100 µg/ml streptomycin (both from Pfizer, Inc., New York, NY), 15 mM HEPES (Sigma Chemical Co., St. Louis, MO), and 10% fetal bovine serum (FBS; Sterile Systems, Inc., Logan, UT). RAW-264, PU-5, and P388D1 macrophage cell lines were grown in HEPES-buffered modified Eagle's medium supplemented as above. J774.1 cells were cultured in DMEM/107. All of these lines were passed three times weekly in 100 mm square Petri dishes.

Membrane preparation. Membranes were prepared according to the method of Thom, et al. (184), with slight modifications. WEHI-3 cells were harvested from spinner culture and washed three times with borate-buffered saline. Membranes were extracted by adding cells dropwise to 20 mM boric acid containing 0.2 mM EDTA. Crude membranes were collected after passing the lysate through glass wool by centrifugation at 12,000 x g for 30 min at 4°C. Pellets

were resuspended in phosphate-buffered saline (PBS) and membranes were purified by centrifugation onto a 35% sucrose cushion at 24,000 x g for 1 hr at 4° C. The sucrose/PBS interface material was collected with a Pasteur pipet and diluted in PBS for a final centrifugation at 100,000 x g for 30 min. All procedures were carried out at 4° C. Membranes were stored in phosphate-buffered saline containing phenylmethylsulfonyl fluoride (PMSF) for up to 3 months at -70° C. Buffers used during the extraction contained the protease inhibitors PMSF (1 mM), leupeptin (1 µg/ml), pepstatin A (1 µg/ml), and aprotinin (10 µg/ml) (all from Sigma). Human erythrocyte membranes (white ghosts) were prepared essentially as described by Dodge et al. (40). Briefly, human erythrocytes from 50 ml blood collected in Alsever's solution were washed three times in isotonic phosphate buffer (310 imOsm; ideal milliosmolarity), pH 7.4 at 4° C. Erythrocytes were packed by centrifugation at 4,000 x g for 20 minutes at 4° C and resuspended in 15 volumes ice cold 20 imOsm phosphate buffer, pH 7.4, to lyse. After incubation for 30 min at 4° C, ghosts were collected and washed in the same buffer four times by centrifugation at 20,000 x g for 40 minutes at 4° C. Ghosts were stored in 20 imOsm phosphate buffer at -70° C.

Interferons. Immunoaffinity-purified (from L-929 cells) mouse interferons alpha (MuIFN-α) and beta (MuIFN-β)

were kindly supplied by Dr. J. L. Pace of our department (129). The specific antiviral activities for the MuIFN-α and -β were 5.5 x 10^7 and 4.8 x 10^8 U/mg, respectively. Recombinant mouse gamma interferon (MuIFN-gamma) was supplied by Schering Corporation (Bloomfield, NJ). The specific activity of this preparation was 2 x 10^6 antiviral units/mg.

MuIFN-gamma (6 µg) was radioiodinated with 300 µCi of carrier-free $Na^{125}I$ (specific radioactivity 16-17 mCi/µg I; Amersham) using chloramine T (71) for 60 seconds at 25°C, and desalted over a 5-ml column of Bio-Gel P6-DG (Bio-Rad Laboratories, Richmond, CA). Specific activities were typically in the range of 10-60 µCi/µg protein, with retention of at least 50% of the biological activity, measured as the capacity to prime macrophages for tumor cell killing (133).

Binding Assays. Binding of ^{125}I-MuIFN-gamma to whole cells (2 x 10^7/ml) was performed in HEPES-buffered modified Eagle's medium containing 5% fetal bovine serum and 20 mM sodium azide (the latter to inhibit endocytosis of the ligand). The assay was terminated abruptly by centrifugation of the cells through a mixture of dibutyl and dioctyl phthalate oils (1.1:1). Binding to either membranes (15-20 µg protein/50 µl) or precipitated liposome/protein complex (see Solubilization and precipitation section, below) was performed at 25°C for 60 minutes in 20 mM HEPES buffer, pH

7.2, containing 150 mM NaCl (HEPES/NaCl) and 2 mg/ml bovine serum albumin (Sigma). Bound ligand was separated from free by filtration through cellulose acetate filters with 0.2 µm diameter pores (MSI, Honeoye Falls, NY). Filters had been presoaked in 50% adult bovine serum to reduce nonspecific binding to the filter.

Preparation of liposomes. Phosphatidylcholine (as L-α-lecithin from egg; Avanti Polar-Lipids, Inc., Birmingham, AL), dissolved in chloroform at 20 mg/ml, was dried under nitrogen and resuspended in HEPES/ NaCl with 2 mM $CaCl_2$ at 2 mg/ml. The suspension was agitated vigorously and stored at 4° C for not more than one month.

Solubilization and precipitation. Membranes were solubilized by resuspending them in HEPES/NaCl containing 10% glycerol, a mixture of protease inhibitors, including 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, and 10 µg/ml aprotinin (Sigma), and indicated concentrations of octyl-β-D-glucopyranoside (Sigma). After mixing at 4° C for 1 hr, the suspension was centrifuged (100,000xg, 4° C, 1 hr) to remove insoluble material. To recover solubilized binding activity (159), the extract was diluted with HEPES/NaCl and a 2 mg/ml suspension of phosphatidylcholine (PC) liposomes (see above) to yield final concentrations of 12 mM OG and 0.4 mg/ml liposomes. Acetone was added to this suspension to a final concentration of 37.5%, after which the entire mixture was centrifuged (13,000xg, 4° C, 30

min). The pellet was assayed for binding, as described above. Protein recovered in association with liposomes was quantified using the bicinchoninic acid (BCA) assay (170) (Pierce Chemical Co., Rockford, IL).

Affinity chromatography. MuIFN-gamma was covalently attached to cyanogen bromide-activated Sepharose 4B, according to the manufacturer's instructions (Pharmacia Fine Chemicals, Piscataway, NJ), using a ratio of 350 µg MuIFN-gamma per ml of swollen gel. The coupling efficiency was routinely 80-85%. For affinity chromatography, soluble extracts were added to MuIFN-gamma-Sepharose that had been equilibrated in HEPES/NaCl containing 40 mM OG and 10% glycerol and incubated for 2 hr at 4°C. Unadsorbed material was removed by centrifugation and washing with the equilibration buffer, followed by washing in the equilibration buffer without HEPES prior to elution.

Results

Binding of gamma interferon to WEHI-3 cells and membranes. Several mouse macrophage cell lines (RAW-264, P388D1, PU-5, J774.1, and WEHI-3) were screened for receptor expression, as measured by specific binding of ^{125}I-MuIFN-gamma. Of these, the myelomonocytic leukemia line, WEHI-3, consistently had the highest number of specific binding sites for MuIFN-gamma. At 50 ng/ml ^{125}I-MuIFN-gamma, for example, WEHI-3 cells (2 x 10^6) bound

17,800 CPM, while P388D1, J774.1, PU-5, and RAW-264 bound 8900, 6000, 2900, and 1900 CPM, respectively. This line was chosen, therefore, as the source of receptor. Equilibrium saturation binding of ^{125}I-MuIFN-gamma to WEHI-3 cells was performed. The data, analyzed by the method of Scatchard (157), indicated the presence of approximately 20,000 receptors per cell, with a dissociation constant (Kd) of 7 nM (Fig. 4-1, top). This extent of receptor expression remained stable after the cells had become adapted to spinner culture with daily passage. Under these conditions, nonspecific binding (i.e., that which remained in the presence of a 100-fold excess of unlabelled MuIFN-gamma) was in the range of 10-20%. The linear characteristics of the binding data suggested that only a single class of binding sites was present. Membranes were prepared from WEHI-3 cells and analyzed for binding activity to determine if binding characteristics of the receptor would change. They were unaffected as, once again, a single class of receptors with a Kd of about 7 nM was obtained (Fig. 4-1, bottom). Receptor recoveries varied slightly among preparations, but were generally 3 picomoles/mg protein.

Solubilization of interferon binding activity. In preparation for enriching the receptor, membranes were solubilized using octyl-β-D-glucopyranoside (OG). This non-ionic detergent permitted the use of a soluble receptor

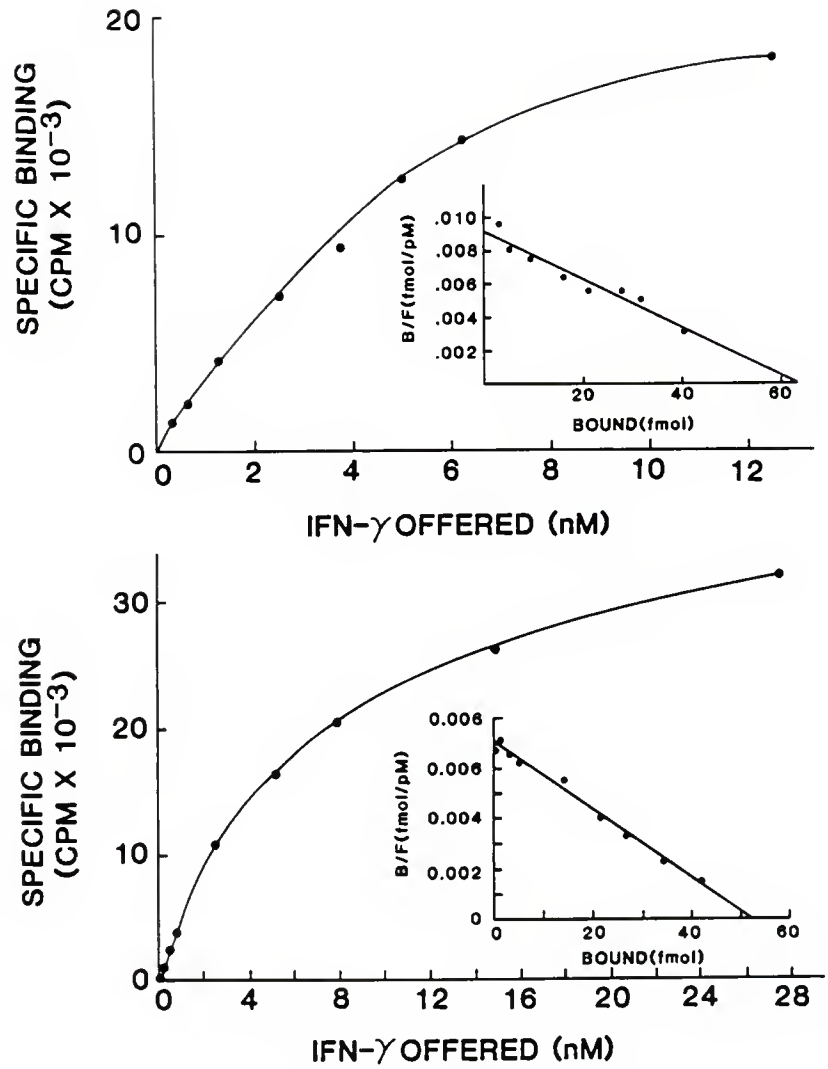

Figure 4-1. Equilibrium saturation binding of ^{125}I-MuIFN-gamma to WEHI-3 cells and plasma membranes.

WEHI-3 cells (top) or plasma membranes (bottom) were incubated with the indicated concentrations of ^{125}I-MuIFN-gamma in the presence or absence of a 100-fold excess of unlabelled MuIFN-gamma to determine specific binding. The whole cell binding assay was performed at 4° C in suspension in the presence of NaN_3. The membrane binding assay was performed at 25° C. Scatchard analysis (insets) indicated dissociation constants of 7 x 10^{-9} M in both cases, with 20,000 receptors/cell and 3 picomoles ^{125}I-MuIFN-gamma bound/mg membrane protein.

assay that has been used before to quantify ligand binding to the solubilized low density lipoprotein (159) and platelet-derived growth factor (35) receptors. Other soluble receptor assays (24,39) were not compatible with the MuIFN-gamma system, primarily due to high nonspecific precipitation or binding of the ligand. When membranes were solubilized with increasing concentrations of OG, interferon-binding activity concomitantly disappeared from the 100,000xg pellet (Fig. 4-2). At the same time, binding activity appeared in the supernatant. The appearance of binding activity in the supernatant was in parallel with recovery of solubilized protein (Fig. 4-2). The amount of binding activity recovered often exceeded that which was lost from the original pellet, i.e., the total binding activity (soluble plus insoluble) at high concentrations of detergent was sometimes as much as two times the amount measured in the original membrane preparation (Fig. 4-2). Solubilization did not reach maximal levels until the OG concentration reached 60 mM.

Properties of solubilized receptor. Repeated washing in aqueous buffer of liposomes that had been co-precipitated with soluble membrane protein did not remove the binding activity for interferon (data not shown). This finding indicated that the binding sites were either integrated into the phospholipid membranes of the liposomes or that their association with them was, at the least,

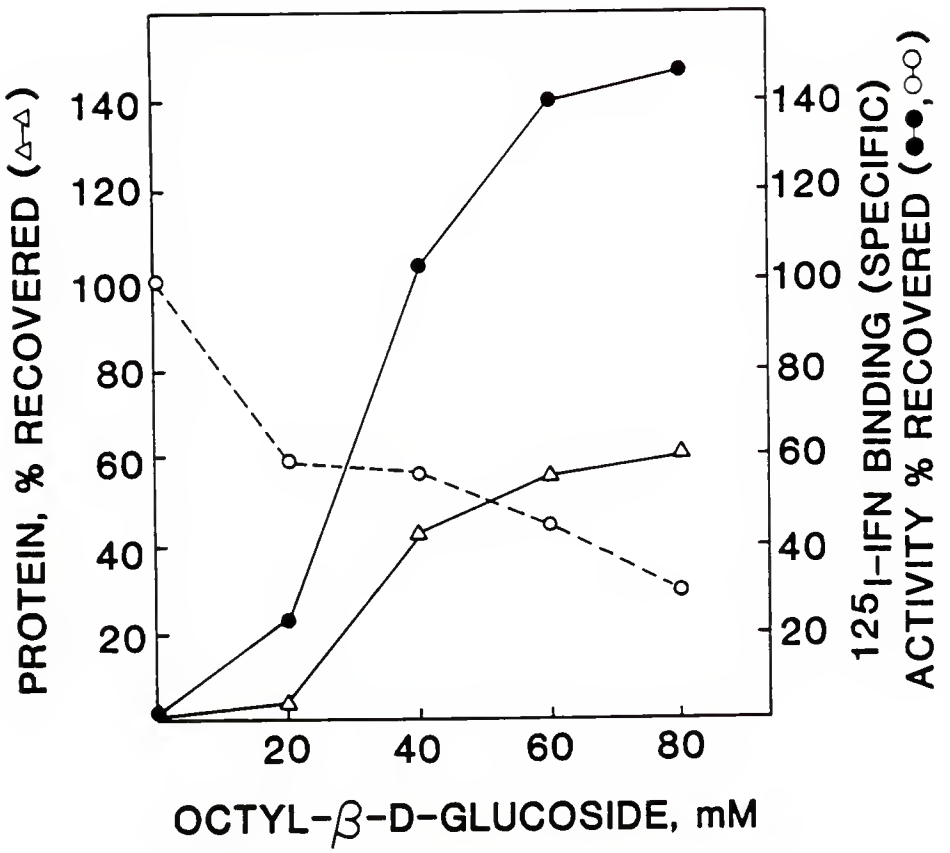

Figure 4-2. Solubilization of interferon binding sites from WEHI-3 membranes.

Membranes were solubilized with the indicated concentrations of octyl-β-D-glucopyranoside at a protein concentration of 1.4 mg/ml. After solubilization for 1 hr at 4° C, soluble material was separated from insoluble material by centrifugation at 100,000 x g for 1 hr. Specific binding of ^{125}I-MuIFN-gamma was determined for soluble (●) and insoluble (o) fractions. Soluble binding was performed after precipitation of the extract with acetone in the presence of liposomes. Protein recovered in the precipitated extract was quantified using the BCA assay (Δ).

strong. Equilibrium saturation binding studies were performed on precipitated extracts to determine if there were any change in the affinity of solubilized binding sites. Scatchard analysis of the data (Fig. 4-3) indicated that the affinity of binding to the receptor in association with liposomes was 13.3 nM, with 6.5 pmol of MuIFN-gamma binding sites estimated/mg of soluble receptor protein (assuming a 1:1 complex between receptor and IFN). Specificity of binding at this stage was maintained with respect to alpha and beta interferons (Fig. 4-4).

The soluble receptor assayed was tested for its specificity for murine IFN-gamma binding sites by solubilizing human erythrocyte membranes (which should not possess these sites) and precipitating them as before. Figure 4-5 compares the extent to which ^{125}I-MuIFN-gamma bound to precipitated extracts from solubilized WEHI-3, human erythrocyte membranes, as well as to PC liposomes alone (i.e., those precipitated from solubilization buffer which contained no membrane extract. The results indicate virtually no specific binding to either PC liposomes alone or to human erythrocyte extracts, while there was the expected amount of binding to precipitated extracts from WEHI-3 membranes.

Use of the liposome assay to quantify enrichment of the receptor. Incubation with MuIFN-gamma-Sepharose beads removed virtually all of the binding activity (>90%) from

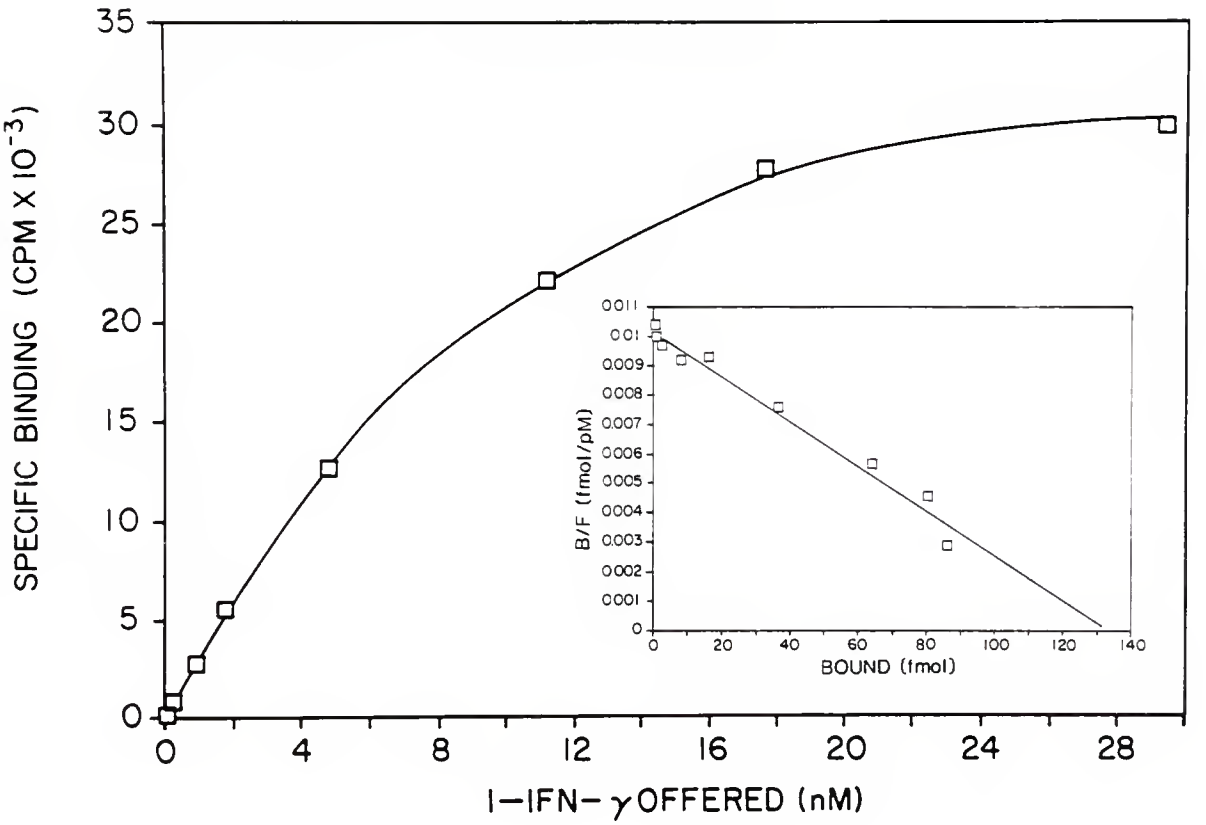

Figure 4-3. Equilibrium saturation binding of ^{125}I-MuIFN-gamma to solubilized receptor.

Membranes were solubilized with 60 mM octylglucoside and the soluble fraction precipitated with liposomes. Precipitates (20 µg protein/tube) were incubated with the indicated concentrations of ^{125}I-MuIFN-gamma in the presence or absence of a 100-fold excess of unlabelled MuIFN-gamma to determine specific binding at 25° C. Scatchard analysis (inset) indicated a dissociation constant of 13.3 nM, with 6.5 picomoles MuIFN-gamma bound/mg protein.

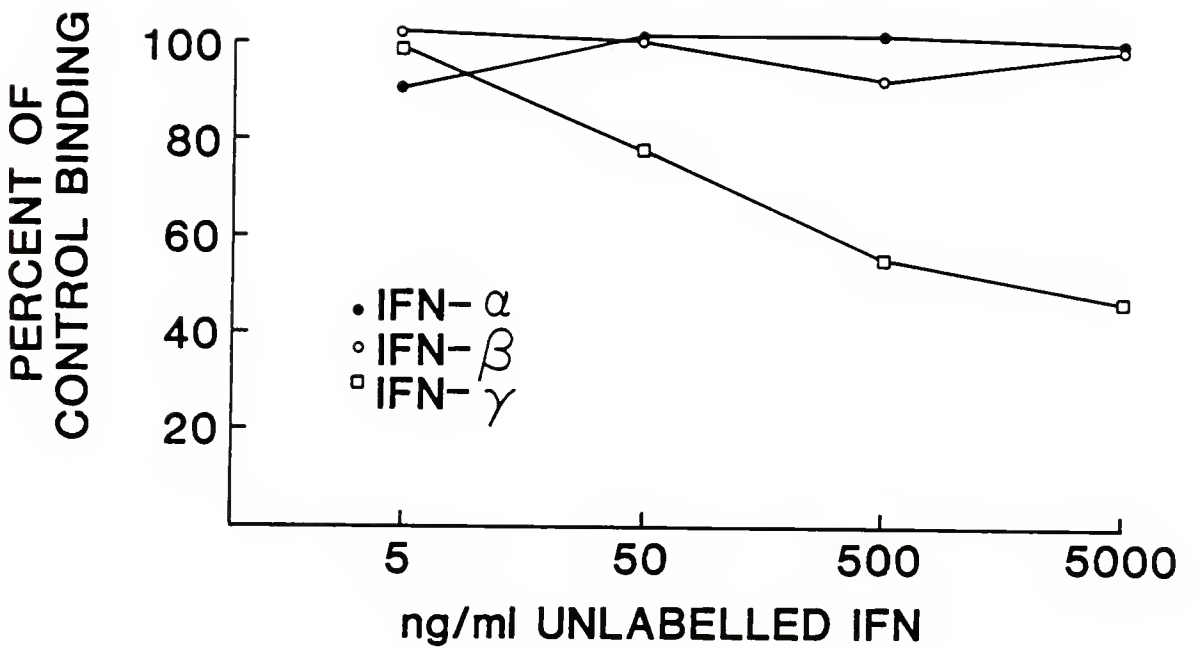

Figure 4-4. Specificity of binding of ^{125}I-MuIFN-gamma to solubilized receptor.

Soluble extracts of WEHI-3 membranes were precipitated with liposomes and incubated with ^{125}I-MuIFN-gamma (50 ng/ml) in the presence of increasing concentrations of α-, β-, or gamma-interferons. Binding was plotted as a percentage of that bound in the absence of interferon.

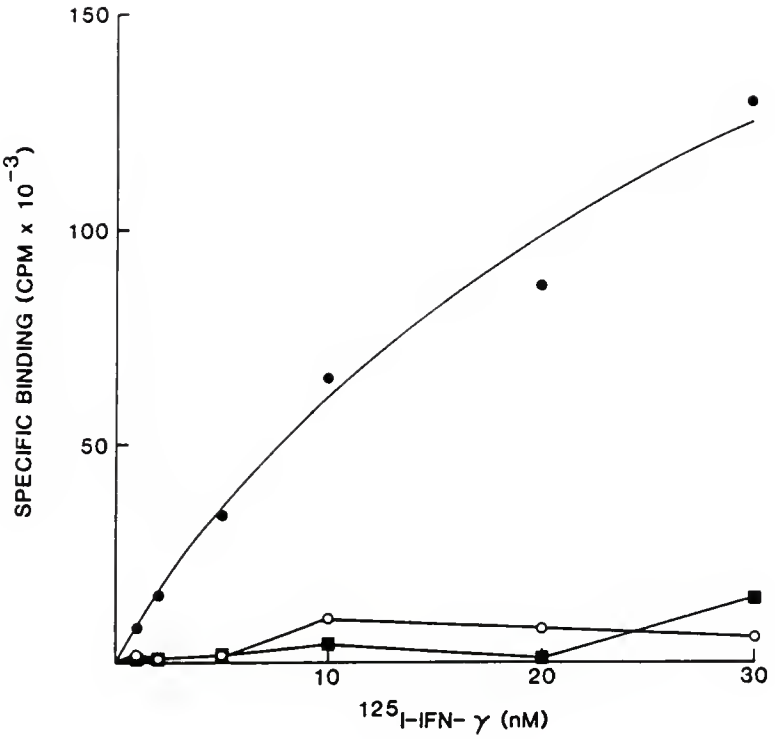

Figure 4-5. Specific binding of ^{125}I-MuIFN-gamma to solubilized WEHI-3 and human erythrocyte membranes, and to PC liposomes alone.

WEHI-3 and human erythrocyte membranes were solubilized in 60 mM octylglucoside at an equivalent protein concentration (5 mg/ml). Soluble extracts or solubilization buffer alone were then precipitated with liposomes and specific binding determined (WEHI-3, ●; human erythrocytes, o; PC liposomes only, ■).

soluble extracts (Table 4-1), with little or no detectable loss in protein content. Differential elution of the beads resulted in approximately 40% recovery of binding activity (Table 4-1). The eluate contained an average of 0.09% of the ^{35}S-labelled proteins that were in the original soluble extract. Based on this, enrichment was estimated in three experiments to be at least 200-fold.

Discussion

Much is known about the phenomenon of macrophage activation by gamma interferon for tumor cell killing, but little is known about the mechanisms that are involved in its induction. One of the most important steps that must be taken to overcome this problem is characterization of the receptor that initiates the process. The work described here is of significance because it provides the means to solubilize and recover this receptor with retention of binding activity. In addition, it provides the basis for quantification of the solubilized receptor, thereby facilitating its purification.

The cells used, WEHI-3, expressed a variable number of receptors depending on the conditions of their culture. Maximal expression, approximately 20,000 sites per cell, was obtained when they were growing exponentially and fed and diluted with fresh medium daily. Under these conditions, the dissociation

Table 4-1. Adsorption of MuIFN-gamma Binding Activity To, and Its Elution From, MuIFN-gamma-Sepharose

Adsorbent	Binding Activity Remaining After Adsorption		Binding Activity Eluted After Adsorption	
	CPM	% Recovery	CPM	% Recovery
None (original extract)	34,400[a]	100	52,085[a]	100
Control Sepharose	27,328[b]	79	ND	ND
MuIFN-gamma-Sepharose	2,008[b]	6	21,184[c]	41

[a] 100 ul of solubilized membrane containing 120 µg protein. Quantification of binding activity was by acetone precipitating it in association with phosphatidylcholine (PC) liposomes, as described under Methods and Materials. CPM are of ^{125}I-MuIFN-gamma bound.

[b] Beads were incubated with soluble membrane extracts for 2 hr at 4°C. After beads were removed by centrifugation, binding activity remaining in supernates was assayed using the PC liposome approach. CPM are of ^{125}I-MuIFN-gamma bound. Control beads were treated and washed as described in Methods and Materials, with the exception that no gamma interferon was included in the reaction mixture.

[c] Binding activity was differentially eluted from MuIFN-gamma-Sepharose beads with 50mM acetate/1 M NaCl (pH 4.5) containing 10% glycerol and 20 mM octyl-β-D-glucopyranoside. Binding activity in the eluate was quantified using the PC liposome approach. CPM are of ^{125}I-MuIFN-gamma bound.

constant for these binding sites on either cells or membranes prepared from them was 7 x 10^{-9} M. Only a single class of binding sites was evident, as has been the case for a variety of other cell types (8,26,27,50,89,98,111, 128,143,156,200), including mononuclear phagocytes (26,27,50,143). No indication of two classes of high and intermediate affinity receptors, as reported by Aiyer et al. (4) was found. This is probably due to our efforts to control internalization of the ligand during the binding assay (low temperature and sodium azide). Internalization during binding can lead to results that can be misinterpreted as suggesting the existence of more than one class of receptor (i.e., a curvilinear Scatchard plot) (4,26). It has been demonstrated that murine gamma interferon is internalized by macrophages at 37° C (29 and Chapter II, this volume).

The change in affinity of the receptor from Kd=7 to 13.3 nanomolar after solubilization could have been due either to alteration of its structure (e.g., denaturation) during extraction or to the change in its lipid environment in the liposomes. In either case, the specificity for gamma, but not alpha or beta, interferon was retained after solubilization. Similar reductions in affinity without alteration of specificity have been reported upon the solubilization of other receptors (25,36,70).

The relatively common (but inconsistent) finding of greater than 100% recovery of binding activity after solubilization (Figure 2) was not due to increased affinity of binding of solubilized receptor, since the affinity actually decreased slightly (see above). A more likely explanation is that there was a variable number of "inside-out" vesicles in the original membrane preparations that resulted in the sequestration of binding sites prior to solubilization. Similar phenomena have been reported to occur during solubilization of the nerve growth factor receptor (141).

The soluble receptor assay was specific for murine IFN-gamma binding sites. No significant binding activity could be recovered from a receptor-negative (i.e., human erythrocyte) cell (Figure 4-5), even at equivalent membrane protein concentrations, while substantial binding activity was recovered from WEHI-3 membranes.

Precipitation of binding activity with phosphatidylcholine liposomes (159) was chosen as the means of recovering solubilized receptor for assay because more conventional soluble receptor assays, such as polyethylene glycol precipitation (39) or filtration over polyethylenimine-treated glass fiber filters (24) were incompatible with the ligand in our system (MuIFN-gamma). This restricted the selection of detergent to octylglucoside, although more complete solubilization was possible with

other detergents (e.g., NP-40). Maximal solubilization with octylglucoside was achieved at a detergent concentration of 60 mM, considerably greater than its critical micellar concentration, but similar to that concentration required to solubilize the receptor for platelet-derived growth factor (35).

Finally, these results show that the recovery of IFN-gamma binding activity in association with liposomes could be used to monitor enrichment and purification of the receptor. Considerable enrichment of IFN-gamma binding activity could be obtained in one step by affinity chromatography on MuIFN-gamma-Sepharose. Eluted material from this matrix should provide sufficiently enriched preparations for immunization and production of antibodies to the receptor that will permit further purification and structural characterization.

CHAPTER V

SUMMARY, DISCUSSION, AND FUTURE DIRECTIONS

The ability of macrophages, when appropriately stimulated, to kill tumor cells selectively has been a subject of intensive research since its original description by Hibbs et al., in 1972 (67). Macrophage activation for tumor cell killing is a model of macrophage biology that reflects the remarkable functional plasticity of these cells in response to the various stimuli in their microenvironment. The phenomenon of macrophage activation has been well characterized (1). Further major advances in understanding the biology of macrophage activation will require studies at the molecular level. The results reported here represent some of the first steps toward delineating the molecular events involved in the induction of macrophage activation for tumor cell killing. These first critical steps include the interactions of stimuli with cell surface structures that induce the tumoricidal state.

The availability of purified, recombinant-derived murine gamma interferon permitted studies of the specific interactions of this molecule with macrophages. In Chapter

II, this ligand was radio-iodinated and used to define specific binding parameters for MuIFN-gamma on various macrophage populations. The results confirmed that mouse macrophages express a single class of specific, high affinity (0.4-4 nM) receptors for MuIFN-gamma. The affinity estimated here was one to two orders of magnitude higher than the first report for murine IFN-gamma receptors by Celada et al. (27), and, in contrast to the only other report of murine macrophage receptors for IFN-gamma (4), only a single class of binding sites was detected. The binding parameters for ^{125}I-MuIFN-gamma confirmed that only a small number of receptors need to be occupied, albeit for a period of at least four hours, for the induction of a tumoricidal response in the presence of sub-activating concentrations of a triggering stimulus. The existence of "spare" receptors has been commonly observed for various ligands (201), and may be related to the stimulation of macrophages through the binding, internalization, and recycling of receptors, as proposed by Celada et al. (29). In contrast to their data, however, macrophages that have been primed or activated (in the presence of LPS) with MuIFN-gamma appear to express significantly fewer receptors (Figure 2-6), suggesting that recycling of receptors to the cell surface may not take place efficiently once the macrophage has responded to the ligand. Alternatively,

receptors may be constantly synthesized and degraded, and the synthesis is reduced upon the response to MuIFN-gamma.

Results of binding analyses on macrophage populations which differ significantly in their responsiveness to activating agents indicate that the response of macrophages for tumoricidal function in these cases is not regulated at the MuIFN-gamma receptor level. Macrophages can also be experimentally manipulated (by trypsinization) to substantially decrease their response to MuIFN-gamma and triggering stimuli without affecting their ability to bind and respond to MuIFN-gamma alone (Chapter III). In all of these cases, the decreased response must either be a result of the loss of response to the triggering stimulus, or to a general loss of the capacity to develop tumoricidal activity at a post-receptor level. In the case of trypsinization, the former would appear to be the case, as the response alterations depend upon the triggering agent used in the induction of activation (Figure 3-6). Similar experiments varying the triggering stimulus might reveal the nature of response differences in the type I treatment and resident/inflammatory models.

The treatment of macrophages with proteases provided insights into cell surface interactions involved in the induction of activation. The receptor for MuIFN-gamma on mouse macrophages was resistant to digestion by trypsin and chymotrypsin, but was susceptible to the broader spectrum

enzyme, Pronase. Trypsin resistance distinguishes the murine from the human receptor (26). It does not necessarily reflect absence of tryptic digestion, but the inability of trypsin action on the ability of macrophages to specifically bind and respond to MuIFN-gamma. This lack of effect of trypsin on the MuIFN-gamma response permitted the dissection of a second level of response that was trypsin-sensitive. The most likely explanation of this sensitivity is that this enzyme affects a cell-surface component required for triggering by either LPS or heat-killed L. monocytogenes (Figure 3-6). Recent evidence has implicated the β-chain of the CR3 (complement receptor type 3; C3bi receptor, LFA-1 (lymphocyte function-associated antigen), and p150,95 family of adhesion-promoting glycoproteins as a putative LPS receptor (204). There is evidence to suggest that the LFA-1 molecule is functionally associated with the trypsin-sensitive tumor cell binding site induced in primed macrophages (180). Direct studies like those of Wright and Jong (204) and the use of specific antibodies to the β-chain of these glycoproteins should reveal the role of this molecule in the response of macrophages to triggering by LPS and other molecules. Polyinosinic:polycytidylic acid appears to interact with a distinct structure, possibly the maleyl-BSA receptor (74), that is trypsin-resistant. The definitive characterization of cell surface structures that interact in a functional

manner with all known triggering stimuli should facilitate a more complete understanding of the triggering process. The data in Chapter III indicate that the actual triggering event for tumoricidal activity can be dissociated from the induction of proteins previously associated with such stimuli. This is not a surprising finding in light of the rapidity of response by which triggering is known to occur. Characterization of triggering stimulus receptors should help to define the rapidly induced events leading to the cytolytic phenotype.

Further significant advances in the understanding of MuIFN-gamma receptor biology and the roles of such a receptor in the induction of activation require its isolation and purification. The results of Chapter IV address the first steps in this endeavor. First, a macrophage cell line (WEHI-3) was found which would provide a relatively rich source of receptor. Plasma membrane preparations from these cells were prepared and found to retain intact receptors. These could then be solubilized with detergent (octylglucoside) to yield receptor in a form suitable for purification. The greatest obstacle for enrichment and purification of the receptor was the development of an assay to detect and monitor the receptor in its soluble state. This was overcome by the use of the liposome-based precipitation assay demonstrated in Chapter IV. Use of this assay permitted the quantification of

soluble receptor upon enrichment by various procedures, the most effective of which was affinity chromatography on immobilized MuIFN-gamma. These advances have allowed the purification of, and preparation of antibodies to, the murine gamma interferon receptor by others in our laboratory; the development of the latter should prove invaluable in the structural characterization of the MuIFN-gamma and the cloning of DNA sequences encoding it. Such information will be essential for the complete understanding of MuIFN-gamma-mediated macrophage responses and should contribute significantly to the delineation of molecular mechanisms of the induction of macrophage activation.

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BIOGRAPHICAL SKETCH

Mark Patrick Hayes was born in Winston-Salem, North Carolina, on April 24, 1959, the youngest of three sons of Donald Michael Hayes and Kathryn Barrier Hayes. After completion of his secondary education at R. J. Reynolds Senior High School in Winston-Salem, Mark attended Davidson College in Davidson, North Carolina, from which he received his Bachelor of Science degree in 1981, with a major in biology. While at Davidson, Mark became a member of the Pi Kappa Alpha fraternity and pursued his musical interests by singing with the Davidson College Male Chorus. Upon graduation from Davidson, Mark worked for a year as a research technician in the Department of Pathology at the Bowman Gray School of Medicine. This work was in the laboratory of Dr. Richard W. St. Clair, and while there he participated in studies on the effects of dietary manipulation on cholesterol metabolism in a primate model for atherosclerosis. Mark spent his summers between undergraduate school semesters in similar research positions.

Mark was married to Robin Ann Belton, also of Winston-Salem, and a graduate of the University of North Carolina at Chapel Hill, on January 2, 1982. In the fall

of 1982, he was enrolled in the Graduate Program in Experimental Pathology, a joint program of the Departments of Pathology, College of Medicine, and Comparative and Experimental Pathology, College of Veterinary Medicine, at the University of Florida. In November of 1982, Mark was awarded a fellowship on a National Institutes of Health Training Grant to support him for the duration of his graduate training. He was admitted to Ph.D. candidacy in the spring of 1984, and pursued his dissertation research in the laboratory of Dr. Stephen W. Russell. Upon completion of his Ph.D., Mark plans to engage in post-doctoral research in immunology, cell and molecular biology.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Stephen W. Russell, Chairman
Professor of Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Alfred F. Esser
Professor of Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Lindsey M. Hutt-Fletcher
Associate Professor of Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Sue A. Moyer
Associate Professor of
Biochemistry and Molecular Biology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 1987

Dean, College of Medicine

Dean, Graduate School

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